T cell-dependent B cell hyperactivity in primary Sjögren's syndrome

Biomarker and target for treatment

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TABLE OF CONTENTS

1

GENERAL INTRODUCTION

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INTRODUCTION TO PRIMARY SJÖGREN'S SYNDROME

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disease, primarily affecting the salivary and lacrimal glands [1]. Inflammation of the glands is accompanied by sicca symptoms, including a sensation of dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia). Predominantly woman are affected by the disease, and the prevalence of diagnosed pSS is estimated at 0.04% of the general population [2]. This prevalence is likely an underestimation, as misdiagnosis of patients with pSS is common due to the large diversity in initial clinical manifestations. In addition to sicca symptoms, more than 50% of patients experience extraglandular symptoms, including chronic fatigue, arthralgia and Raynaud's phenomenon. Extraglandular manifestations may also involve the lungs, skin, kidneys and nervous systems [1]. Current treatment options for pSS are only symptomatic. In the past years, several immunomodulatory treatment approaches for pSS were evaluated, but none of them have been approved yet. Thus, there is an unmet need for treatment options that can halt or cure this disease.

Pathophysiology of pSS

The lack of efficacy of treatment modalities so far may be explained by the fact that the pathophysiology of pSS is multi-facetted and not completely understood. Both environmental and genetic factors probably contribute to disease initiation, and the few gene polymorphisms that have been associated with pSS are related to components of both innate and adaptive immune systems [3]. In particular genes that are involved in the NF-kB pathway, the interferon (IFN) signaling pathway, lymphocyte signaling, and antigen presentation have been associated with SS [4]. The strongest risk loci were found in the HLA region, with the top variants residing in the HLA-DR and HLA-DQ regions. Outside the HLA region, the strongest association was found at the Interferon Regulatory Factor 5 (IRF5) gene locus [3]. This gene is involved in IFN signaling and B cell differentiation towards plasma cells (reviewed by [4]). The involvement of IFN signaling in pSS pathogenesis is further reflected by the presence of a type I IFN signature (i.e., overexpression of type I IFN inducible genes) in 55-60% of the patients [5,6]. This signature is associated with higher systemic disease activity, higher levels of autoantibodies, and higher transcript levels of B cell activating factor (BAFF) in monocytes [5]. Also, stimulation of cultured salivary gland epithelial cells with IFN resulted in upregulation of BAFF expression by epithelial cells [7]. IFN-induced BAFF expression may link innate and adaptive immune activation in pSS.

Histopathology

A hallmark of pSS is focal, periductal infiltration of T lymphocytes and B lymphocytes in salivary and lacrimal gland tissues, accompanied by loss of glandular architecture and function [8]. Lymphocytes can even infiltrate the epithelium and together with proliferative metaplastic epithelial cells form characteristic lymphoepithelial lesions (LELs), which are most pronounced in the parotid gland [9]. Furthermore, periductal infiltrates can become organized in lymphoid tissue with segregated T and B cell areas and high endothelial venules. In approximately 25% of pSS patients, germinal centers (GCs) arise within this tertiary (ectopic) lymphoid tissue [10]. GCs facilitate local generation of (auto)antibody-producing plasma cells and memory B cells [11].

The periductal localization of the infiltrates illustrates the importance of the epithelium in the disease process. This epithelium is not only target of the disease but also exerts important immunological functions including cytokine production and antigen presentation [12]. In addition to periductal infiltration of the target tissue and LEL formation, a shift in the plasma cell compartment is a third histological hallmark of pSS. This shift is mostly in favor of IgG-expressing plasma cells. Increased numbers of salivary gland IgG-producing plasma cells likely contribute to the circulating levels of autoantibodies in pSS patients [13].

Extraglandular manifestations

Extraglandular manifestations of pSS can be differentiated in peri-epithelial or immune complex-mediated manifestations. Examples of peri-epithelial manifestations are interstitial nephritis and obstructive bronchiolitis. Cutaneous vasculitis, peripheral neuropathy, and glomerulonephritis are examples of immune complex-mediated manifestations [14,15]. Patients with peri-epithelial manifestations usually have a more stable disease than patients with immune complex mediated-manifestations. In addition, hematologic abnormalities, such as leucopenia (including lymphopenia), anemia and thrombocytopenia, are common in pSS patients [16,17]. Patients with pSS also have a 5- to 16-fold increased risk for the development of malignant B cell lymphoma (reviewed by [18]). Eventually, 5-10% of patients develop a lymphoma in the salivary glands, particularly of the mucosa-associated lymphoid tissue (MALT) type [19,20]. These lymphomas may well reflect the characteristic B cell hyperactivity seen in these patients (see below).

The systemic activity of pSS is strongly associated with several serologic abnormalities, including low C4 levels, hypergammaglobulinemia, cryoglobulinemia and higher levels of rheumatoid factor and anti-SS-A(Ro)/SS-B(La) autoantibodies [17,21]. These serologic signs may therefore predict the evolution of extraglandular symptoms and identify patients in need for systemic treatment.

T cell-dependent B cell hyperactivity

Increased levels of autoantibodies, together with the presence of hypergammaglobulinemia and cryoglobulins, reflect the ongoing T cell-dependent B cell hyperactivity in pSS patients. An important cytokine axis that appears to be involved in this T cell-dependent B cell hyperactivity is the IL-6/IL-21 axis. IL-6 is overexpressed in saliva, tears and minor salivary glands of pSS patients (reviewed by [22]). One of the many effector functions of IL-6 is direct and/or indirect stimulation of B cell proliferation and differentiation into plasma cells (reviewed by [23]). Indirect stimulation of B cells by IL-6 is mediated via differentiation of naïve $CD4^+$ T cells into T follicular helper (Tfh) cells and induction of IL-21 production by these cells in response to IL-6 [24]. IL-21 is a potent inducer of plasma cell formation and is involved in GC B cell selection [25,26]. Therefore, the IL-6/IL-21 axis is thought to play a pivotal role in B cell activation in pSS patients. Furthermore, together with TGF-β, IL-6 may contribute to immunopathology by the induction of Th17 cell differentiation [27].

Despite the notion that B cell hyperactivity is a central event in the disease process, their specific pathogenic role remains controversial. Current evidence indicates that this role goes beyond autoantibody production [28], as antigen presentation and cytokine production by B cells might be significantly involved in pathogenesis of pSS. Activity of the IL-6/IL-21 cytokine axis and subsequent stimulation of both antibody-dependent and antibody-independent B cell functions may result in a pro-inflammatory amplification loop, which enhances infiltration of lymphocytes and non-lymphoid mononuclear cells to the target tissues of pSS patients. Together with the autoreactivity, apoptosis and possibly also intrinsic defects of the glandular epithelium, the inflammation contributes to dysfunction or even destruction of the exocrine glands and other tissues and finally in worsening of the clinical symptomatology. Expanding knowledge of the various cell types and mediators involved in immunopathology of pSS has opened new ways for the development of selective treatment modalities in pSS. Vice versa, application of the newly developed treatment modalities may help to understand the pathogenesis of the disease.

Treatment of pSS

Conventional synthetic immunomodulatory drugs in pSS

The use of conventional immunomodulatory drugs in pSS is largely extrapolated from its effectiveness in other autoimmune diseases, such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE). Examples of frequently prescribed 'off-label' drugs in pSS are prednisone, hydroxychloroquine, and azathioprine. Prednisone is a synthetic corticosteroid with broad immunosuppressive effects. In pSS patients, low-dose prednisone is used for the treatment of arthritis and cutaneous symptoms,

based on clinical experience. High-dose prednisone is used to treat severe systemic manifestations of pSS. Evidence regarding the use of prednisone for the treatment of pSS is limited, as trials were small and specifically designed to assess the effect on sicca features. Prednisone seems to improve hypergammaglobulinemia and lymphopenia [29], which are common biological abnormalities in pSS patients, associated with systemic disease activity [17,21].

Hydroxychloroquine (HCQ) is a disease-modifying antirheumatic drug (DMARD) that suppresses endosomal activation of Toll-like receptor (TLR)7 and TLR9 [30,31]. Consequently, HCQ impairs innate immune responses, including pro-inflammatory cytokine (e.g. interferon) production. In pSS patients, HCQ is used for the treatment of articular and skin involvement based on the efficacy observed in SLE and RA. However, two placebo-controlled trials did not show a clinical benefit of HCQ in pSS patients [32,33]. The suggested application of HCQ for treatment of articular involvement in pSS was not confirmed by these trials. Too few patients with skin involvement were included to draw any conclusion concerning this manifestation. No clear benefit has been demonstrated of other conventional immunomodulatory drugs that were evaluated in pSS patients (reviewed by [34]). These drugs include azathioprine, methotrexate and mycophenolic acid. Treatment with leflunomide showed only modest clinical efficacy in a phase II open-label study, but did ameliorate leucocytoclastic vasculitis in three pSS patients [35]. The high rate of adverse events reported for many of these conventional immunomodulatory drugs raises concerns about whether they should be prescribed off-label in pSS.

Biologic immunomodulatory agents in pSS

Anti-TNF agents were the first biologic drugs evaluated in pSS patients. Unexpectedly, at that time, these biologicals did not show efficacy [36,37]. In 2005, rituximab, a chimeric anti-CD20 monoclonal antibody, was introduced in pSS for the treatment of pSS patients with MALT lymphoma. Thereafter, several open-label and placebo-controlled trials followed to evaluate the efficacy of rituximab in the treatment of pSS, which has been a hotly debated issue ever since [38]. In addition to B cell depletion therapy with rituximab, other potential drugs for pSS that target B cells directly are anti-CD22 antibodies (e.g. epratuzumab), anti-CD40 antibodies (e.g. CFZ533), and antibodies that bind to the BAFF receptor (e.g. VAY736). BAFF signaling is involved in survival, activation and differentiation of B cells (reviewed by[39]). Next to B cells, additional targets for treatment have emerged as a result of advanced understanding of pSS pathogenesis. None of these biologicals have yet been approved for the treatment of pSS.

Given the recognized role for T cells in pSS pathogenesis, targeting of these cells is also considered to be a rational therapeutic option in pSS. Abatacept is a fully human

fusion molecule of IgG-Fc and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Abatacept prevents the co-stimulatory interaction between antigen-presenting cells, including B cells, and T cells. Selective co-stimulation modulation by abatacept is presumed to inhibit full T cell activation and T cell-dependent B cell activation. Additionally, other strategies that block co-stimulatory pathways (e.g. anti-CD40) or cytokine binding (e.g. anti-IL-6R) are currently being evaluated in pSS. Targeted synthetic DMARDs that inhibit intracellular BCR- or cytokine receptor signaling, such as Bruton's tyrosine kinase (BTK) inhibitors, phosphoinositide 3-kinase (PI3K) inhibitors and Janus kinase (JAK) inhibitors, have become available as well (illustrated in Figure 1). In summary, many possible treatment options for pSS are underway and have the potential to halt disease progression and ameliorate symptoms. However, to realize patient-tailored treatment we need additional biomarkers that can predict (progression of) systemic disease activity and treatment response.

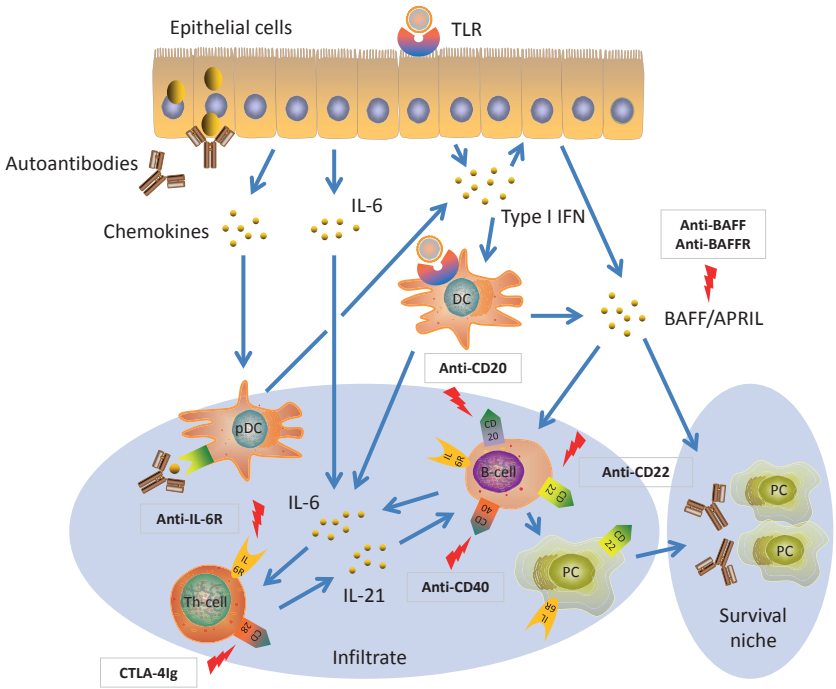

FIGURE 1 | Targets for systemic treatment with biologic agents in primary Sjögren's syndrome (pSS). Different aspects of the inflammatory response in target tissues of pSS are shown. The pathogenic role of epithelial cells in the disease process is reflected by infiltration of mononuclear cells in epithelial tissues. Furthermore, epithelial cells secrete inflammatory proteins, such as type I IFNs, BAFF, IL-6 and chemokines. There is a subsequent migration of various cell types to the tissue and then all elements to carry out (auto-) immune responses are in place. Key targets for treatment and biologic agents that have been investigated are illustrated. APRIL: A proliferation-inducing ligand; BAFF: B cell activating factor; DC: Dendritic cell; PC: Plasma cell; pDC: Plasmacytoid dendritic cell; Th-cell: T-helper cell; TLR: Toll-like receptor.

AIM OF THIS THESIS

The aim of this thesis was to assess the role of T cell-dependent B cell hyperactivity in pSS, both as a biomarker of disease (activity) and as a target for treatment. Biomarkers in blood and tissue were studied in a variety of patient cohorts, and effects of immunomodulatory therapies on the immune system of treated patients were evaluated.

In the studies described in **part one** of this thesis, we investigated the relevance of several T cell and B cell-related biomarkers of pSS, and discussed their role in disease initiation, clinical manifestation, and/or disease progression. In **chapter 2** we reviewed the role of Th17 cells in pSS pathogenesis, also in relation to their plasticity, i.e. ability to adapt different effector functions. In the study described in **chapter 3a** we show that in addition to the elevated frequencies of Tfh cells, the ratio between Tfh cells and T follicular regulatory (Tfr) cells is altered in pSS patients, already at the time of diagnosis. Furthermore, we show that Tfr cells from pSS patients express lower levels of CTLA-4, a receptor involved in immune suppression. These alterations may have important implications for establishment of B cell hyperactivity. In the study described in **chapter 3b**, we evaluated the potential role of the Tfr/Tfh ratio and frequency of activated Tfh cells in blood as biomarkers of pSS. In **chapter 4** we moved to the B cell side and revealed the phenotype and gene expression profile of $FcRL4^{+}$ B cells, a subtype of mucosa-associated B cells. These cells were found in close association with the ductal epithelium in the inflamed salivary glands, and are thought to be the cell type from which MALT lymphomas arise. Our findings help to elucidate their role in pSS pathogenesis. In the study described in **chapter 5** the pathological role of B cells was assessed from a more clinical perspective. Possible applications of serum immunoglobulin free light chains (FLC) as biomarkers of MALT lymphoma and systemic disease activity in pSS are shown. We also provide evidence for a role of FLCs as biomarkers of treatment response, making the study described in this chapter a stepping stone to the second part of this thesis.

In **part two** of this thesis we focused on the effect of immunomodulatory drugs on the immune system of treated pSS patients. We went back from bedside to bench to study which cell types and cytokines were affected by treatment and were important for treatment response. In the study described in **chapter 6** we show that the effects of B cell depletion therapy with rituximab are not restricted to B cells, but that this treatment also significantly affects the T cell compartment, in particular Tfh cells. **Chapter 7** provides an overview of available data on the efficacy of rituximab in pSS, including clinical and biological effects, and underlines the value of this treatment for a subgroup of pSS patients. The study described in **chapter 8** illustrates that blockade of CD28-mediated T cell co-stimulation by abatacept has significant effects on T cell-dependent B cell hyperactivity. The research reported in **chapter 9** focused on the

expression of the BCR signaling molecule Bruton's tyrosine kinase (BTK) by B cells. We show that a subgroup of pSS patients has increased BTK levels across different B cells subsets, indicating a lower threshold for activation. In addition, we were able to show that abatacept treatment reduced BTK levels, illustrating the positive feedback loop between T cell and B cell activation. Finally, the results presented in this thesis are summarized and discussed in **chapter 10**. Based on our findings we consider ways to accelerate establishment of effective treatment modalities for pSS.

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PART I

T CELL-DEPENDENT B CELL HYPERACTIVITY: BIOMARKER OF DISEASE?

2

TH17 CELLS IN PRIMARY SJÖGREN'S SYNDROME: PATHOGENICITY AND PLASTICITY

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ABSTRACT

Th17 cells play an important physiological role at mucosal barriers, and are involved in inflammatory responses to pathogens. Th17 cells and their signature cytokine IL-17 are also present in salivary gland lesions of primary Sjögren's syndrome (pSS) patients and can be elevated in their peripheral blood. In pSS patients, clear correlations between increased Th17 cell activity and symptoms of the disease have not been found, but Th17 cells may contribute to disease progression, for example by supporting autoreactive B cell responses. In mouse models of pSS, Th17 cells play an important role in pathogenesis, particularly at disease onset, when there is a disturbed balance between T effector and T regulatory cells. Studying the pathogenicity of Th17 cells in humans is complicated due to the plasticity of this cell subset, allowing them to obtain different effector functions depending on the local environment. Th17 cells can develop towards Th17.1 cells, producing both IL-17 and IFN-γ, or even towards Th1-like cells producing IFN-γ in the absence of IL-17. These effector subsets may be more pathogenic than bona fide Th17 cells. Co-expression of IFN-γ by Th17 cells has been shown to promote chronic inflammation in several autoimmune diseases and may also contribute to pSS pathogenesis. In line with the noticeable role of IL-17 in pSS mouse models, interference with Th17 cell generation, recruitment or effector functions (e.g. IL-17 inhibition) can prevent or ameliorate disease in these models. Therapies targeting Th17 cells or IL-17 have not been tested so far in pSS patients, although treatment with rituximab seems to lower local and systemic IL-17 protein levels, and to a lesser extent also chemokine receptor-defined Th17 cells. In this review we discuss current knowledge of pathogenicity and plasticity of Th17 cells in human pSS and murine models of pSS. We postulate that plasticity towards Th17.1 cells in pSS may enhance pathogenicity of Th17 cells at the main target sites of the disease, i.e. salivary and lacrimal glands.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease, primarily affecting the salivary and lacrimal glands. Oral and ocular dryness, fatigue and pain are predominant symptoms of pSS. The disease is clinically heterogeneous and many extraglandular organs can be involved during the course of the disease [1]. The pathophysiology of pSS is multi-facetted and not completely understood. Both environmental and genetic factors are likely involved in disease initiation, and the few gene polymorphisms that are associated with pSS are related to components of both innate and adaptive immune systems [2]. No polymorphisms in genes encoding salivary or lacrimal components have been identified. Involvement of the adaptive immune system is evident in the affected exocrine glands of pSS patients, where main histopathological findings include periductal focal infiltration of mononuclear cells, largely consisting of $CD4^+$ T cells and B cells [3]. These periductal infiltrates can be organized into ectopic lymphoid structures with segregated T and B cell areas. In approximately 25% of the patients, these structures contain germinal centers, which promote local expansion of (auto)antigen-specific (memory) B cells [4,5]. The occurrence of ectopic germinal centers, together with hypergammaglobulinemia and presence of autoantibodies underlines the important role of B cell hyperactivity in pSS pathogenesis [6]. It is, however, important to note that $CD4^+$ T cells predominate the periductal infiltrates in patients with mild lesions [3]. Growing evidence suggests that the crosstalk between $CD4^+$ T cells and B cells forms a crucial step in pSS pathogenesis and a suitable target for treatment [7,8].

Different $CD4^+$ T cell subsets seem to contribute to pSS pathogenesis, including T helper 1 (Th1) cells, follicular T helper (Tfh) cells and T helper 17 (Th17) cells, although the relative importance of each subset remains a matter of debate. After the first discoveries of a link between Th17 cells and autoimmunity, several human and murine studies investigated the role of Th17 cells in pSS pathogenesis, as summarized in Table 1. In 2008, the first studies showed that IL-17, the signature cytokine of Th17 cells, is present within lymphocytic infiltrates of minor salivary gland tissue from pSS patients [9,10]. Presence of IL-17 was predominantly observed in CD4+ T cell-rich areas of the periductal infiltrates [10]. Also IL-17 mRNA levels were elevated in minor salivary glands of pSS patients, compared with non-SS sicca patients [11]. Subsequent studies focused on the presence of Th17 cells within the glands. However, there is not a single marker that identifies Th17 cells exclusively. In current literature, Th17 cells have been identified either by expression profiles of their signature cytokines IL-17 and IL-22, by the expression of chemokine receptors (CCR6, CCR4, CD161, podoplanin) and/or by means of transcription factors (RORγ, STAT3). To complicate matters further, Th17 cells can acquire functional characteristics of regulatory T (Treg) cells, Th1 cells and Tfh cells and even can downregulate IL-17 production, illustrating the plasticity of this cell subset [12].

TABLE 1 | Evidence for the involvement of Th17 cells in pSS pathogenesis.

Reference	Publication	Study population	Key observations related to Th17 cells
Human			
[8]	Verstappen et al., 2017	pSS patients before and after abatacept treatment	Patients with pSS have elevated frequencies of circulating Th17 cells (CCR6+CCR4+), compared with controls. These cells are not affected by abatacept treatment.
[9]	Nguyen et al., 2008	pSS patients	Protein expression of IL-17 and IL-23 in lymphocytic foci in minor salivary glands of pSS patients. IL-17 levels in serum and saliva of pSS patients comparable to non-SS sicca patients.
[10]	Sakai et al., 2008	pSS patients	Protein expression of IL-17 in minor salivary glands was predominantly found in CD4+ T cell areas, but also co-localized to some extent with CD8+ T cells and ductal epithelial cells.
[11]	Katsifis et al., 2009	pSS patients	Local IL-17 protein and mRNA levels, together with IL-6 and IL-23 mRNA, increase with progression of lesion severity in minor salivary glands of pSS patients. Plasma IL-17 levels were significantly higher in pSS patients, compared with controls.
[13]	Ciccia et al., 2014	pSS patients before and after RTX treatment	Salivary gland expression of IL-17, but not of IL-23p19 and p-STAT3, decreased by rituximab treatment.
[14]	Liu et al., 2017	pSS patients	IL-17A conjunctival mRNA and protein expression in tears higher in pSS, compared with non-SS group with dry eye disease.
[15]	Ciccia et al., 2012	pSS patients	IL-22 is present in minor salivary gland tissue of pSS patients and Th17 cells are a major source of this cytokine.
[16]	Blokland et al., 2017	pSS patients	Percentages of peripheral IL-17-producing CD4+ T cells were similar between pSS patients and controls. CCR9+ Th-cells produced IL-17 upon antigen and IL-7 stimulation.
[17]	Verstappen et al., 2017	pSS patients before and after RTX treatment	Frequency of IL-17-producing CD4+ T cells in PBMCs from pSS patients at baseline was similar to controls, but these cells significantly decreased by rituximab treatment, together with serum levels of IL-17.
[18]	Bikker et al., 2012	pSS patients	Ex vivo and IL-7-induced IL-17A production is similar in pSS patients and controls
[19]	Kwok et al., 2012	pSS patients	Higher frequency of IL-17-producing CD4+ T cells in PBMCs from pSS patients, compared with controls.
[20]	Pollard et al., 2013	pSS patients	Several Th17-related cytokines (IL-17, GM-CSF, IL-1β) were significantly elevated in pSS patients, compared with controls.
[21]	Reksten et al., 2009	pSS patients	Higher levels of Th17-associated cytokines in pSS patients with germinal center (GC) formation in their salivary glands, compared with GC-negative patients.
[22]	Alunno et al., 2013	pSS patients	IL-17-producing CD4-CD8- T cells are expanded in PBMCs from pSS patients, are also present in minor salivary glands and are resistant to in vitro dexamethasone suppression.
[23]	Fei et al., 2014	pSS patients	Glandular IL-17 protein expression increased with progression of lesion severity. CD4+IL-17+ cells in peripheral blood of pSS patients and serum IL-17 were significantly increased, compared with controls.

TABLE 1 | Continued

Reference	Publication	Study population	Key observations related to Th17 cells
Mouse			
[9]	Nguyen et. al., 2008	C57BL/6.NOD-Aec1Aec2 mice	IL-17A, IL-17R and IL-23 expression in salivary glands when infiltrates occur, Tbet is increased in the pre-disease phase.
[24]	Voigt et. al., 2016	C57BL/6.NOD-Aec1Aec2 x IL-17 KO mice	IL-17 deficient C57BL/6.NOD-Aec1Aec2 mice are protected against disease development.
[25]	Wanchoo et. al., 2017	C57BL/6.NOD-Aec1Aec2 mice	TCR repertoires of Th1 and Th17 cells in salivary gland infiltrates are restricted.
[26]	Lin et. al., 2015	C57BL/6J and IL-17 KO mice with ESS	Th17 cells are increased in salivary gland peptide induced disease. IL-17 deficient mice are protected, and transfer of Th17 cells in IL-17 deficient mice restores disease phenotype.
[27]	Iizuka et. al., 2015	RORγt Tg mice and RAG KO mice	RAG deficient mice develop pSS phenotype upon transfer of RORγt overexpressing CD4+ T cells, but not when these cells are IL-17 deficient.
[28]	Lee et. al., 2012	C57BL/6.NOD-Aec1Aec2 with IL27 expression in salivary glands	IL-27 expression through rAAV2-*IL27 vector injection*, which induces Th1 and inhibits Th17 cells is most effective after onset of glandular disease
[29]	Contreraz-Ruiz et. al., 2017	TSP1 KO mice with TSP1 peptide treatment	Treatment of TSP1 KO mice with TSP1 derived peptide increases Treg cells and reduces Th17 cells, and attenuates disease symptoms.
[30]	Coursey et. al., 2017	NOD.B10.H2^{b}	Treg cell function is hampered and Treg cells cells start to produce IL-17 and IFNγ.
[31]	Iizuka et. al., 2010	M3R KO and RAG KO mice	Transfer of M3R deficient splenocytes in RAG deficient mice leads to Th17.1 infiltration in salivary glands and pSS like symptoms.
[32]	Tahara et. al., 2017	M3R KO and RAG KO mice with anti-RORγt treatment	RORγt antagonist treatment after transfer of M3R deficient splenocytes into RAG deficient mice reduces both IL-17 and IFNγ in spleen and LN.
[33]	Nguyen et. al., 2010	C57BL/6J with IL-17A expression in salivary glands	IL-17A expression through Adenovirus 5 cannulation in salivary glands leads to pSS-like phenotype

In this review we will discuss current knowledge of Th17 cells in pSS pathogenesis and mouse models of pSS, including their phenotype, localization, function and correlation with clinical features of the disease. We will focus on the relation between pathogenicity and plasticity of Th17 cells and postulate that plasticity towards Th1-like cells in pSS may enhance pathogenicity of Th17 cells at the main target sites of the disease, i.e. salivary and lacrimal glands.

Role of Th17 cells in pSS

Th17 cells play an important physiological role at mucosal sites of healthy individuals. The main effector cytokines of Th17 cells are IL-17 an IL-22. These cytokines support the epithelial barrier integrity by stimulation of tight junction protein formation [34], and

IL-22 has an important role in epithelial cell survival and proliferation [35]. Th17 cells also act as first defense against microbes by stimulating the production of antimicrobial peptides and chemokines to attract leukocytes when the epithelial barrier is breached [36]. Initially, activation and polarization of Th17 cells may be initiated by dendritic cells in lymph nodes draining the salivary and lacrimal glands, whereas in later phases of the disease this may also happen locally in the inflamed glandular tissue. These dendritic cells secrete Th17 cell polarizing cytokines, including TGF-β and IL-23 (Figure 1). Ductal epithelial cells of the glands may also produce cytokines important for Th17 polarization, such as IL-1β [37]. Activated Th17 cells promote inflammation by stimulating release of pro-inflammatory cytokines in the inflamed exocrine glands, including IL-6 and TNF, by virtue of IL-17 and IL-22 secretion and its binding to their receptors expressed on stromal and epithelial cells [38] (Figure 1). Expression of IL-17R was observed in a neoplastic parotid gland cell line [10], and is likely also expressed by ductal epithelial cells in pSS patients. IL-17 was also shown to induce matrix metalloproteinase 1 (MMP-1) and MMP-3 release from synovial fibroblasts in rheumatoid arthritis, which may cause tissue destruction [39]. In salivary gland tissue of pSS patients, particularly MMP-9 expression is increased and is associated with acinar damage [40]. Interleukin-17 also promotes MMP-9 production by epithelial cells [41].

In addition to their role in tissue inflammation, Th17 cells also may contribute more specifically to autoimmune processes by the following mechanisms (i) supporting isotype class switching upon B cell receptor stimulation, both via IL-17 and IL-21 production [42,43], (ii) regulating glycosylation of autoreactive antibodies [44], (iii) affecting trafficking of B cells within the GC resulting in disturbed selection of B cells and formation of autoantibodies [45] and (iv) supporting formation of ectopic lymphoid tissue and ectopic germinal centers (GCs) [45–47] (Figure 1). Whether these functions of Th17 cells are involved in pSS pathogenesis is currently unknown. There is some support for a role of IL-22 in ectopic lymphoid tissue formation in pSS. Administration of luciferase-encoding replication-defective adenovirus (Ad5) through intraductal cannulation into the salivary glands of C57BL/6 mice leads to lymphocytic infiltration of these glands, and ectopic lymphoid tissue formation. Knockout or blockade of IL-22 in this model impaired ectopic lymphoid tissue formation [48]. This was probably caused by reduced IL-22-mediated CXCL12 and CXCL13 production by stromal cells in these IL-22 deficient animals. In summary, numerous potential effector functions of Th17 cells may contribute to pathogenesis in autoimmune conditions in general, and pSS in particular [49].

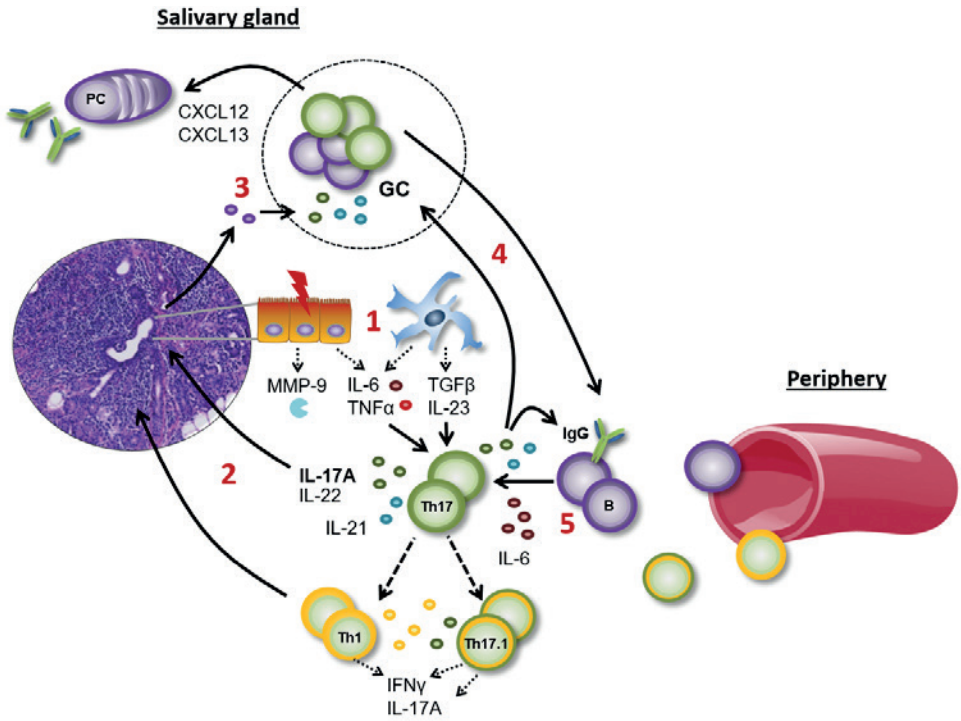

FIGURE 1 | Role of Th17 cells in primary Sjögren's syndrome (pSS) patients. 1) Environmental factors activate epithelial cells and dendritic cells (in blue). These cells secrete pro-inflammatory cytokines and present antigens, resulting in activation of Th17 cells. 2) Th17 cells infiltrate the salivary gland and may differentiate towards Th17.1 cells or Th1 cells. Pro-inflammatory cytokines (IL-17A, IL-22, IFNγ) are secreted by these cells and bind to their receptors expressed on stromal and epithelial cells. Tissue inflammation is exacerbated and more pro-inflammatory factors are secreted by epithelial cells. 3) CXCL12 and CXCL13 are expressed by stromal and epithelial cells and, together with antigen presentation by follicular dendritic cells, can induce germinal center (GC) formation in salivary glands. 4) The GC generates plasma cells producing autoantibodies and memory B cells switched to IgG, which is stimulated by IL-17 and IL-21. 5) B cells in secondary lymphoid organs and salivary glands produce IL-6, further stimulating formation of Th17 cells.

Th17 cells and glandular inflammation in pSS

Interleukin-17 protein and mRNA, as well as cells expressing the Th17-associated transcription factor RORγ, are present in minor salivary gland tissue of pSS patients, mainly in CD4+ T cell-rich areas [9–11,13,14]. IL-17 is also present in saliva and tears from pSS patients, and in tears, levels are higher compared with non-SS sicca controls [14,50]. Although it is likely that Th17 cells are the main source of IL-17 in the inflamed exocrine glands, γδ T cells, NK cells, innate lymphoid cells (ILCs) including lymphoid tissue inducer cells, and $CD8^+$ T cells are also potent sources of IL-17 [49]. Double negative (CD4-CD8-) T cells and CD8+ T cells that are positive for IL-17 are actually present in minor salivary glands of pSS patients, albeit in low numbers. Immunohistochemical analysis initially

suggested that also mast cells were a source of IL-17 in inflamed salivary glands [13]. Recent findings show, however, that mast cells do not produce IL-17 themselves, but actively capture IL-17 by endocytosis [51].

The number of IL-17-positive cells and IL-17 mRNA levels in minor salivary gland biopsies correlate with focus score, a measure of glandular inflammation [11]. Another Th17 cell-associated cytokine that is present in salivary gland tissue of pSS patients is IL-22, which seems to co-localize with mononuclear cells and ductal epithelial cells [15]. The same study showed that, after in vitro stimulation, IL-22 is mainly co-expressed by IFN-γ- or IL-17-producing CD4+ T cells isolated from minor salivary glands and only a small proportion of CD4+ T cells expressed IL-22 alone [15]. The IL-22 receptor (IL-22R) is usually expressed by nonhematopoietic cells at barrier surfaces [52]. However, only few ductal and acinar epithelial cells in the salivary glands of pSS and non-SS sicca patients seem to express IL-22R, and aberrant protein expression of IL-22R was observed among infiltrating mononuclear cells in pSS patients [53]. The nature and function of this IL-22R expression on mononuclear cells is, however, unclear.

The developmental origin of IL-17- and IL-22-expressing T cells in salivary glands is not exactly known, and both local differentiation from naïve CD4+ T cells as well as recruitment of Th17(-like) effector cells from the peripheral blood may contribute to the local pool of IL-17- and IL-22-expressing cells (Table 2). Naïve T cells can differentiate locally into Th17 cells in the presence of antigen presenting cells (APCs) and the essential cytokines IL-6 and TGF-β [54]. IL-6 is present in salivary gland tissue and saliva of pSS patients and local IL-6 expression increases with a higher focus score [55]. TGF-β is also produced in salivary gland tissue of both healthy individuals and pSS patients [11]. Th17 cell differentiation is further amplified by IL-21 and this cytokine is abundantly expressed in the glandular infiltrate of pSS patients [56,57]. In addition to IL-6 and TGF-β, also the pro-inflammatory chemokines CXCL9 and CXCL10 may play a role in local polarization of Th17 cells. Activated $CD4^+$ T cells may express CXCR3 and ligation of CXCR3 not only leads to upregulation of Tbet, the transcription factor driving Th1 cell differentiation, but also to RORγ expression and Th17 cell formation [58]. In this context it is relevant to mention that CXCL9 and in particular CXCL10 are secreted in high quantities by ductal epithelial cells from pSS patients in response to IFN-γ [59] and likely also to IFN-α [60].

Besides local differentiation of naïve cells and polarization of Th1 cells, Th17 cells can also be recruited from the circulating pool of Th17 cells by chemokines that are secreted in the salivary glands. An important pathway for direct recruitment of Th17 cells to the inflamed tissue is via the CCL20/CCR6 signaling axis [16,61]. CCL20 is not only important for recruitment of Th17 cells, but also for activation of these cells, as binding of CCL20 to CCR6 induces calcium influx in Th17 cells [62]. CCL20-mRNA transcripts were, however, only detected at low levels and in few pSS patients as revealed by qPCR [63,64]. Thus, the role for CCL20 in the recruitment of Th17 cells to the salivary glands seems limited.

Th17(-like) cells may, however, also be attracted by other chemokines, such as CCL25/CCR9. Recently it was shown that IL-17-producing CCR9$^+$ T cells home in small numbers to the inflamed salivary gland under the influence of CCL25 [16]. Another signaling axis that may contribute to recruitment of both naïve and central memory (Th17) cells consists of CCR7 and its ligands CCL19 and CCL21, all of which are highly expressed in salivary gland tissue of pSS patients [65,66].

TABLE 2 | Ligands and receptors that promote Th17 cell polarization, recruitment and maintenance in (inflamed) human salivary glands.

Expressed by naïve / activated T cell	Ligand	Expressed by	Effect	Reference
Local polarization of naïve CD4$^+$ T cells into Th17 phenotype				
IL-6 receptor	IL-6	APC, ductal epithelial cells	IL-6 and TGFβ together promote Th17 differentiation by upregulating RORγt and IL-23R expression on Th17 cells	[11,54,55]
TGFβ receptor	TGFβ	APC		
IL-21 receptor	IL-21	Tfh cells/Th17 cells	amplification of Th17 differentiation	[56,57]
CXCR3	CXCL9 / CXCL10	ductal epithelial cells (among others)	upregulation of Tbet and RORγt on T cells	[58–60]
Expressed by Th17 cell	**Ligand**	**Expressed by**	**Effect**	**Reference**
Recruitment of Th17 cells to the salivary glands				
CCR6	CCL20	salivary gland epithelial cells (low expression)	homing of Th17 cells to salivary glands and activation of these cells	[61,62,64]
CCR7	CCL19 / CCL21	salivary gland stromal cells (high expression)	homing of naïve T cells and central memory Th17 cells to salivary glands	[65,66]
CCR9	CCL25	inflamed salivary gland tissue (epithelial cells)	homing of CCR9+IL-17+ T cells to salivary glands	[16]
Maintenance of Th17 cells in salivary glands				
IL-23 receptor	IL-23	APC	expansion and maintenance of Th17 cells and production of cytokines	[9,11,49]
IL-7 receptor	IL-7	salivary gland stromal cells	maintenance of pathogenic Th17 cells	[68,69]
IL-15 receptor	IL-15	salivary gland epithelial cells	maintenance of pathogenic Th17 cells	[68,70]

Not only pro-inflammatory cytokines that induce or amplify Th17 cell differentiation, but also cytokines that are important for homeostasis of Th17 cells may contribute to Th17-mediated pathology in inflamed tissue. IL-23 is important for expansion and maintenance of Th17 cells by STAT3 activation and is present in glandular infiltrates [9,11,49]. Production of IL-23 by macrophages is at least in part mediated by the activation of interferon regulatory factor 5 (IRF5) [67]. Interestingly, polymorphisms of the IRF5 gene locus are associated with pSS and may enhance IL-23 production

[2]. IL-7 and IL-15 can also sustain pathogenic Th17 cells, which is mediated by STAT5/Akt signaling [68]. Elevated levels of IL-7 are observed in minor salivary glands of pSS patients, compared with non-SS sicca patients, and IL-7 is largely produced by stromal cells in the glands [69]. IL-15 can be produced by salivary gland epithelial cells of pSS patients in response to TLR2 stimulation in vitro [70].

Taken together, the inflamed exocrine glands in pSS constitute a microenvironment that enables local polarization and recruitment of (precursor) Th17 cells. Although their contribution to the disease is not clear yet, local Th17 cells can acquire several effector functions that are potentially pathogenic.

Th17 cells and systemic inflammation in pSS

In addition to glandular Th17 cell activity, also circulating Th17 cells and serum levels of IL-17 have been studied in the past decade in pSS, but with conflicting results [9,11,17–21]. Some studies report an increase in circulating Th17 cells and/or serum levels of IL-17, whereas others do not find a difference between pSS patients and healthy controls. It should be noted that different definitions of Th17 cells were used in these studies.

Recently, we found in two independent study cohorts that proportions of circulating Th17 cells, as defined by their chemokine receptor expression profile (CD4+CD45RA-FoxP3-CXCR5-CXCR3-CCR4+CCR6+), were increased in pSS patients compared to healthy controls [8,17]. Both studies included patients with moderate systemic disease activity, as measured by ESSDAI, the EULAR Sjögren's Syndrome Disease Activity Index (median ESSDAI scores in these study cohorts: 11 and 8, respectively). Despite this increase in chemokine-receptor defined Th17 cells in these patients, proportions of circulating CD4+IL-17+ T cells were not elevated [17], consistent with a previous report [18]. The relative increase in Th17 cells, as defined by chemokine receptor expression, was not observed when comparing pSS patients with non-SS sicca patients in a diagnostic cohort that included patients clinically suspected with pSS (Verstappen & Kroese, unpublished data). In this cohort, systemic disease activity in pSS patients was low (median ESSDAI score = 4). These findings indicate that elevated levels of Th17 cells are possibly only seen in pSS patients with moderate to high systemic disease activity.

Alternative definitions of Th17 cells have been adopted to study the prevalence of Th17 cells in peripheral blood of pSS patients. For example, expression of the C-type lectin CD161, in combination with RORγ, the master transcription factor required for generation of Th17 cells and IL-17 production, has been used [71]. In pSS patients, CD4+CD161+RORγ+ T cells were increased and this increase correlated positively with anti-SSA/SSB autoantibody status and serum IgG level, but not with systemic disease activity, as measured by ESSDAI [72]. Recent findings show that, in addition to typical CCR4+CCR6+ Th17 cells, also circulating 'Tfh-like' CCR9+CD4+ and CXCR5+CD4+ T

cells from pSS patients are capable of producing IL-17 [16]. Regarding the latter Tfh-like subset, a fraction of these cells appears to co-express CCR6, and thus may also be considered as a Th17 cell subset. These CD4+CXCR5+CCR6+ T cells were elevated in peripheral blood of pSS patients [73]. Lastly, circulating double negative (CD4-CD8-) T cells, which consist largely of γδ+ T cells, are a potential source of IL-17 in pSS patients [22]. Also these double negative T cells that produce IL-17 are expanded in peripheral blood of pSS patients [22]. The chemokine receptor profile of double negative T cells still needs to be defined.

Even though definitions of Th17 cells vary, these cells thus seem to be increased in peripheral blood of pSS patients. Likely, both circulating and local Th17 cells contribute to serum levels of IL-17, although, as mentioned before, also other cell types are able to produce this pivotal Th17 cell cytokine. Nearly all studies showed increased IL-17 (i.e. IL-17A) levels in serum of pSS patients. However, a correlation between serum IL-17 levels and disease activity has not been reported [9,11,20,21,23,74]. Reksten et al. showed that serum levels of IL-17 were higher in pSS patients with GCs in their minor salivary gland biopsies compared to GC-negative patients [21]. Subsequently they observed that serum IL-17 levels correlated positively with levels of anti-Ro/SSA and anti-La/SSB autoantibodies, but not with clinical features of the disease [74]. These findings, together with our observations that circulating Th17 cells are increased only in patient cohorts with moderate-to-high systemic disease activity, but not in patients with low systemic disease activity, indicate that numbers of circulating Th17 cell and levels of serum IL-17 are associated with disease severity and/or with certain stages of the disease. In line with this notion, a positive correlation between disease duration and levels of circulating Th17 cells and serum IL-17 was observed in mouse models of pSS [75].

Th17 cells in mouse models of pSS

Mouse models of pSS are very useful to study aspects of the disease that otherwise cannot be addressed. Although these models often only mimic part of the pathology found in pSS patients, they do give important insights in the role of individual cells or cytokines, and provide the opportunity to study disease kinetics.

The most extensively used animal model to study pSS is the C57BL/6.NOD-*Aec1Aec2* mouse. These mice harbor two susceptibility loci that promote a spontaneous pSS-like autoimmune phenotype, featuring salivary and lacrimal gland dysfunction leading to decreased saliva production and ocular inflammation [76]. In these mice, RORγt, IL-17 and IL-17R mRNA expression were found in the salivary (submandibular) glands [9]. Elevated IL-17 and IL-17R expression was also seen at the ocular surface [77]. Correspondingly, Th17 cells were present in the immune infiltrates in salivary and lacrimal glands of

affected mice [24,77]. However, only low levels of IL-17 were found in serum of these mice [9]. Despite these low serum IL-17 levels, IL-17 seems to play an important role in pSS-like disease in this model. This is illustrated by the observation that IL-17-deficiency in C57BL/6.NOD-*Aec1Aec2* mice significantly reduces the pro-inflammatory response in the salivary glands and restores normal secretory function, particularly in female animals [24]. In addition, these mice exhibit an altered specificity of auto-antibodies compared to IL-17-sufficient C57BL/6.NOD-*Aec1Aec2* mice, illustrating the role of IL-17 in promoting autoreactive B cells responses. This effect is probably mediated by affecting the numbers of both GC B cells and plasma cells [24]. These data suggest that IL-17 is particularly pathogenic at the site of inflammation. This is further supported by a model in which SS-non-susceptible C57BL/6J mice received local IL-17A gene transfer in the salivary glands, resulting in glandular inflammation, autoantibody production and decreased saliva production [69]. In addition to pro-inflammatory roles of IL-17 in C57BL/6.NOD-*Aec1Aec2* mice, a recent study also shows that T cell receptor repertoires of Th1 and Th17 cells in the salivary glands are limited compared to wild type controls, particularly in female animals [25], suggesting they may be skewed towards recognition of autoantigens.

In a second mouse model of pSS, disease is induced by immunization with autoantigenic peptides derived from salivary glands [78]. Also in these mice, Th17 cells are abundantly present in the salivary gland infiltrates and draining lymph nodes, and are the main IL-17 producing T cell subset [78]. In parallel, these mice have high serum levels of IL-6 and TGFβ, which are essential cytokines for Th17 differentiation. Importantly, IL-17-deficient mice immunized with salivary gland peptides are completely protected from disease development and adoptive transfer of Th17 cells (polarized in culture) to these mice restores the autoimmune phenotype [26]. Also a third mouse model, in which RORγt is overexpressed, illustrates the importance of Th17 cells in development of pSS-like disease [27]. These mice exhibit increased IL-17 production by T cells and concomitantly pSS-like features including salivary and lacrimal gland inflammation and autoantibody production [27]. Increased expression of CCR6 was found on splenic $CD4^+$ T cells in these mice, and the ligand for CCR6 (i.e. CCL20) was abundantly expressed in the salivary glands, enabling homing of circulating Th17 cells to these glands [27].

These models not only reveal that Th17 cells are crucial cells for development of pSS-like disease, but also give important clues about their relevance at different time points of disease onset and progression. In the C57BL/6.NOD-*Aec1Aec2* mice, IL-17, IL-23 and RORγt expression increase when the infiltrates arise in the salivary glands, whereas they drop again after development of full-blown disease [9]. These findings suggest that Th17 cells may play a local temporal role at early stages of the disease. However, before the function of Th17 cells becomes apparent, Th1 cells appear to be involved. Even before infiltrates are formed in the salivary glands, levels of Tbet, the transcription

factor driving Th1 cell differentiation, are increased in submandibular glands, in line with the crucial role for IFNγ in the pre-clinical onset of disease in NOD mice [9,79]. This temporal balance between Th1 and Th17 cells in the glandular tissue may determine the development of the autoimmune phenotype. This is further illustrated by gene therapy of these C57BL/6.NOD-*Aec1Aec2* mice with IL-27, a cytokine that promotes Th1 and inhibits Th17 development. Initiation of treatment after disease onset, i.e. at a time point when Th17 cells are thought to play a role, is more effective than treatment before disease onset, i.e. when Th1 cells are involved [28]. Also in the salivary gland peptide-immunized model, first Th1 cells are increased in the salivary glands, and later on Th17 cells predominate [26].

Taken together, there is strong evidence in mice that Th17 cells are a driving force in the pathogenesis of pSS(-like) disease. The pSS mouse models further indicate that Th17 cells and IL-17, are particularly involved in the early phase of disease, a finding that may be more challenging to confirm in pre-clinical disease in humans.

Th17/Treg imbalance in pSS

Autoimmune diseases are frequently linked to an altered Th17/Treg ratio and commitment to one of these lineages is tightly regulated by distinct signaling molecules [80]. Available evidence indicates that there is, however, no imbalance in proportions of effector Th17 cells and Treg cells in pSS patients, as both subsets are equally increased in the periphery of pSS patients with moderate systemic disease activity [8]. Furthermore, the numbers of both Th17 cells and FoxP3+ cells in minor salivary gland tissue correlate positively with focus score/grade of inflammation [11,81]. It is not known though whether the population of FoxP3+CD4+ T cells in pSS patients is functionally normal and is able to suppress effector T cells.

Although these observations strongly argue that there is no Th17/Treg imbalance in human pSS, several mouse models suggest that an imbalance between Th17 cells and Treg cells could underlie the development of this disease (Figure 2). This imbalance may be a result of increased IL-6 in the inflammatory environment. TGFβ in the absence of IL-6 induces Treg differentiation, but TGFβ and IL-6 together promote Th17 differentiation [82]. In C57BL/6.NOD.*Aec1Aec2* mice, Treg cells are decreased compared to wild-type control mice in the lacrimal gland already at an early pre-clinical disease age, when Th17 cell numbers and IL-17A expression are increased [77]. Consistent with these findings, transient depletion of Treg cells in NOD mice led to increased salivary gland infiltrates [83]. A role for Th17/Treg imbalance in disease induction is further illustrated in mice lacking thrombospondin-1 (TSP1), an important activator of latent TGFβ in vivo [84]. These mice spontaneously develop ocular inflammation accompanied by dry eye symptoms and anti-SSA and anti-SSB antibodies [85]. Increased splenic Th17 cells and lacrimal IL-17 protein levels in these mice were accompanied by a decrease in splenic

Treg cells [85]. In vivo administration of TSP1-peptide to TSP1 knock-out mice induced formation of FoxP3+ Treg cells, and decreased Th17 cells, attenuating symptoms of disease [29].

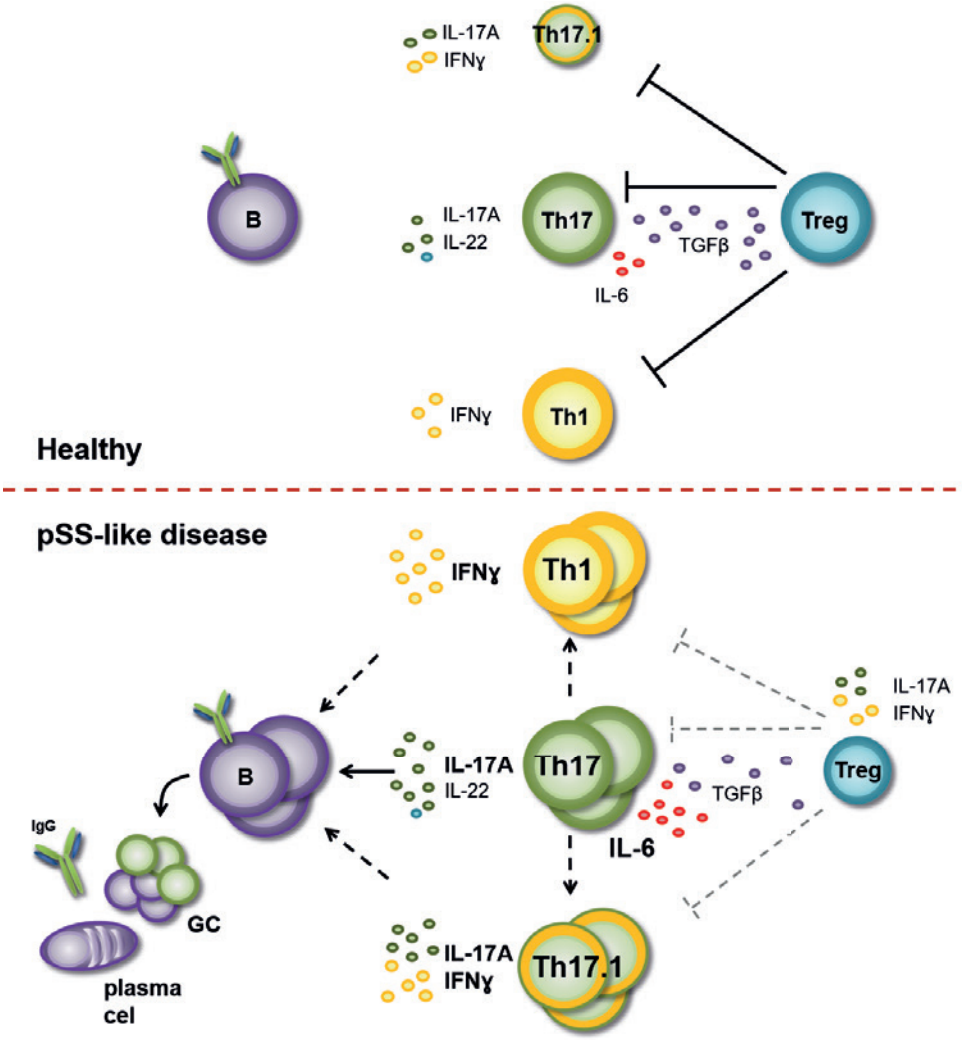

FIGURE 2 | Insights on Th17 cell plasticity from pSS mouse models. In a healthy situation, there is no inflammation in the salivary glands and at the ocular surface. Treg cells control Th1 cells, Th17 cells and the small number of Th17.1 cells present in the body. However, in mice with pSS-like disease, IL-6 levels increase, shifting the balance between Treg cells and Th17 cells. Treg cells are reduced in number, lose their regulatory capacity and sometimes start producing IL-17 and IFNγ. Simultaneously, the number of Th17 cells increases, and these cells can convert to IL-17 and IFNγ producing Th17.1 cells, or to IFNγ single producing Th1-like cells. Together, these cells can promote germinal center formation, and support differentiation of B cells into class-switched plasma and memory cells.

In a different NOD model bearing an altered MHC region (NOD.B10.H2^{b} mice), animals spontaneously develop ocular surface disease upon aging. In these aged mice, FoxP3+ Treg cells aberrantly co-express Tbet and RORγt and produce IFN-γ and IL-17. At the same time, aged Treg cells in NOD.B10.H2^{b} mice exhibit lower suppressive capacity compared to Treg cells from young mice. Transfer of CD4^{+}CD25^{+} Treg cells from these aged mice into T and B cell-deficient (RAG1-deficient) animals induced a similar phenotype of periductal inflammation in the lacrimal glands as transfer of CD4^{+}CD25^{-} T helper cells [30]. These results confirm that Treg cells can acquire pro-inflammatory features associated with Th1 and Th17 cells.

Together, these murine models illustrate that not only the enhanced pro-inflammatory features of Th17 cells can promote disease, but that changes in Treg cells, both in number or function, may contribute to disease progression. Functional assays with human Treg cells from pSS patients could clarify whether decreased suppressive capacity or even pro-inflammatory capacity of Treg cells also plays a role in the development of disease in patients.

Plasticity of Th17 cells in pSS

Both in humans and mice, Th17 cells are not a "fixed" subset, but can acquire features from, or differentiate towards, other effector types, i.e. Th1 and Treg cells [86]. The transformation of typical Th17 cells towards Th17.1 cells is most intensively studied, especially in the context of autoimmunity [87] (Figure 2). These Th17.1 cells co-express CXCR3 and CCR6 and produce both IL-17 and IFN-γ.

Plasticity of Th17 cells in humans is, however, a relatively unexplored field. In patients with Crohn's disease, Th17.1 cells are pathogenic and promote chronic inflammation [88]. Furthermore, in patients with multiple sclerosis Th17.1 cells reacted strongly against self-antigens[89]. The factors that drive this plasticity in humans are not fully understood, but some indications may come from a murine model of experimental autoimmune encephalomyelitis. In these mice, transformation of Th17 cells to both IFN-γ-single producing Th1 cells and IFNγ/IL-17 double producing Th17.1 cells was driven by high IL-7 expression [90]. Interestingly, in salivary gland tissue of pSS patients, IL-7 is abundantly present [69], and may drive the plasticity of Th17 cells to IFN-γ single or double producing Th17.1 cells. Besides plasticity of Th17 cells, plasticity of other effector T cell subsets may also contribute to the pathology seen in pSS patients. For example, it has been shown in mice with experimental autoimmune encephalomyelitis (EAE) that Tfh cells can aberrantly express IL-17, and these IL-17-producing Tfh cells could augment the formation of autoreactive B cells by stimulating ectopic germinal center formation and impairing chemotactic migration of B cells out of the germinal center [47]. Aberrant expression of IL-17 by Tfh cells may also play a role in later phases of pSS pathogenesis when germinal center containing ectopic lymphoid tissue is present.

The possible contribution of Th17 cell plasticity to pathogenicity in pSS is further illustrated by a Sjögren mouse model driven by an immune response against the M3 muscarinic acetylcholine receptor (M3R) [91]. Under physiological conditions, cholinergic stimulation of these receptors leads to an increase of saliva secretion. Immunization of M3R-deficient mice with M3R peptides induces a strong immune response that results in formation of autoantibodies directed against M3R, that block the cholinergic stimulation and lead to reduced saliva production. Such blocking autoantibodies against these receptors have also been described in human pSS patients [91,92]. Besides autoantibody formation, the M3R immunized mice exhibit an increase in IL-17A and IFNγ producing Th17.1 cells in the spleen [31]. Adoptive transfer of splenocytes from these mice into T- and B-lymphocyte deficient animals induced severe pSS-like disease with anti-M3R autoantibody formation and Th17.1 cells infiltrating the salivary glands associated with decreased saliva production [31]. Treatment of these mice with a RORγt antagonist after the transfer of splenocytes, reduced both IL-17 and IFNγ *in vivo*, and partially abrogated disease [32]. These data suggest that Th17 cells could co-produce IL-17 and IFNγ, or that Th17 cells might convert to Th1 cells post-transfer. Although this model is not completely equivalent to pSS pathogenesis, it does show many similarities with human disease, including inflammation specifically of the salivary and lacrimal glands, but not of the intestines or liver, and a similar cellular composition of mononuclear infiltrates in the glands.

In summary, although the data are scarce, they indicate that plasticity of Th17 cells towards more pathogenic Th17.1 cells or Th1 cells may contribute to disease progression in pSS.

Effect of treatment on Th17 cells/IL-17 in pSS

Immunomodulatory treatment of pSS patients may provide important insights into the role of various cell types in pathogenesis. One of the first biological DMARDs that was clinically tested in pSS patients was the TNF-alpha inhibitor etanercept. Markers of activation on B cells and CD4+ T cells were not significantly altered by etanercept treatment, in line with a lack of clinical benefit [93]. Plasma IL-17 levels were also unaffected [11]. Subsequently, several studies assessed the efficacy of B cell depletion therapy with rituximab. Although the clinical benefits are a matter of debate [94], many biological parameters are affected, including Th17 cell-related biomarkers [95]. Rituximab treatment resulted in decreased IL-17 protein expression in minor salivary gland tissue of pSS patients, despite the finding that factors that are important for maintenance of Th17 cell , viz. pSTAT3 and IL-23, were not altered [13]. Dendritic cells and macrophages are major sources of IL-23 and these cells are likely not affected by B cell depletion therapy [57]. In addition to reduced IL-17 expression in the salivary glands, we found decreased frequencies of circulating IL-17+CD4+ T cells and to a smaller

extent also chemokine receptor-defined Th17 cells after rituximab treatment [17]. The decrease of IL-17+CD4+T cells over time correlated with decreasing levels of IgG and autoantibodies, suggesting that IL-17 and autoantibody formation are somehow related. Also serum levels of IL-17 decreased in this study [17]. In a previous study, we found that serum IL-6 levels were also significantly reduced by rituximab treatment [20]. We therefore postulated that the effect of B cell depletion therapy on Th17 cells and IL-17 production is mediated by depletion of IL-6-producing B cells [17]. As mentioned before, IL-6 supports Th17 cell differentiation from naïve T cells and is important for the induction of RORγ and IL-17 [96]. In summary, rituximab affects Th17 cells locally and systemically, although the mechanism of this effect and its contribution to amelioration of disease remains to be established.

As T cell activation and crosstalk between B cells and CD4+ T cells are important features in pSS pathogenesis, T-cell co-stimulation appears to be a suitable target for treatment. Abatacept, which limits CD28-mediated co-stimulation and thereby activation of T cells, was able to reduce systemic disease activity (ESSDAI scores) in pSS patients in a small open-label study [97]. Although the fraction of circulating Tfh cells was significantly reduced by treatment, circulating Th17 cells (CD4+CD45RA-FoxP3-CXCR5-CXCR3-CCR4+CCR6+) were not affected. Also serum levels of IL-17 did not change at a group level, but two patients with the highest baseline levels did show a reduction in serum IL-17 [8]. Apparently, the clinical efficacy of abatacept is not the result of a significant effect on the Th17/IL-17 axis. It is not known yet whether IL-17-producing cells in glandular tissue of pSS patients are affected by abatacept treatment.

So far IL-17-targeting therapies have not been tested in pSS patients. A case report from a patient with psoriasis and pSS treated with ustekinumab, a monoclonal antibody directed against the p40 protein subunit shared by IL-12 and IL-23, showed beneficial effects not only on cutaneous disease, but also on joint involvement [98]. Effects on other pSS-related symptoms, such as dryness, were not reported. A placebo-controlled trial with the IL-6R antagonist tocilizumab is currently ongoing in pSS (NCT01782235). As naïve CD4+ T cells express IL-6R and IL-6 signaling is important for Th17 differentiation, an effect of tocilizumab on Th17 cells is expected and these results may provide more insight into the contributions of Th17 cells to disease activity.

CONCLUSION

Both human and mouse studies clearly indicate that Th17 cells/IL-17 producing T cells are involved in local inflammation in pSS and SS-like disease. Their contribution to systemic disease is more enigmatic. Th17 cells are elevated in the periphery of a subgroup of pSS patients and higher systemic Th17 cell activity (serum IL-17 level, CD161+RORγ+CD4+ cell frequency) correlates with increased autoantibody titers. However, it remains to be

established if Th17 cells contribute directly to pathogenesis of human pSS. A pathogenic role for Th17 cells is more evident in mouse models of SS, where Th17 cells appear to play a key role in development of the autoimmune phenotype. Pathogenicity of Th17 cells in pSS is possibly linked to plasticity of this cell subset. In particular plasticity towards Th17.1 cells, co-expressing IL-17 and IFN-γ (and CCR6 and CXCR3) may support chronic inflammation and B cell activation in pSS patients (Figure 1&2). Furthermore, mouse models indicate that the major contribution of Th17 cells to disease pathology may be temporal, early and locally in affected tissues. Future studies are needed to clarify Th17 cell phenotypes in glandular infiltrates and to address their contribution to disease onset and progression.

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3A

T FOLLICULAR REGULATORY CELLS FROM PATIENTS WITH PRIMARY SJÖGREN'S SYNDROME EXPRESS DECREASED LEVELS OF CTLA-4

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Work in progress

ABSTRACT

Objective

Humoral immune responses rely to a large extent on T follicular helper (Tfh) cells. T follicular regulatory (Tfr) cells are important regulators of Tfh cells. Proportions of circulating Tfh (cTfh) cells are increased in primary Sjögren's syndrome (pSS) patients compared with healthy controls (HCs). Tfh cells probably play a critical role in the enhanced activation of B cells in pSS. This study aimed to assess the frequency and phenotype of cTfh cells and circulating Tfr (cTfr) cells in pSS, as well as their relation to B cell activity and systemic disease activity.

Methods

Sixty-eight pSS patients (66 female, mean age 50) classified according to the ACR-EULAR criteria and 24 HCs (23 female, mean age 43) were included. Cryopreserved peripheral blood mononuclear cells were analyzed by flow cytometry to assess the frequency of cTfh cells ($CD45RA^-FoxP3^-CXCR5^+PD\text{-}1^+$) and cTfr cells ($CD45RA^-FoxP3^+CXCR5^+PD\text{-}1^+$). Median expression levels of CTLA-4 per cell were also measured.

Results

Frequencies of both cTfh cells and cTfr cells were elevated in pSS patients compared with HCs ($P<0.001$). Circulating Tfr cells were even more increased than cTfh cells, resulting in significantly higher cTfr/cTfh ratios in pSS ($P<0.001$). Frequencies of cTfh and cTfr cells correlated positively with serum levels of IgG and CXCL13, and systemic disease activity. In pSS patients, expression levels of CTLA-4 in cTfr cells were significantly decreased compared with HCs ($P<0.001$). Lower expression levels of CTLA-4 in cTfr cells tended to be associated with an increase in cTfh cells ($\rho=-0.382$, $P=0.07$).

Conclusion

The cTfr/cTfh ratio is increased in pSS patients and frequencies of both subsets correlate with measures of B cell activity and systemic disease activity. Circulating Tfr cells from pSS patients express lower levels of CTLA-4, suggesting that despite their increased frequencies, their ability to suppress is impaired. A reduced suppressive capacity of Tfr cells may contribute to the expansion of Tfh cells and consequently to B cell hyperactivity in pSS.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by lymphocytic infiltration of salivary and lacrimal glands. B cell hyperactivity is a key feature of pSS pathogenesis, and is, among others, reflected by elevated serum IgG levels [1]. T follicular helper (Tfh) cells, an effector subset of $CD4^+$ T cells, probably play a critical role in the enhanced activation of B cells. An important mechanism of B cell help by Tfh cells is the production of IL-21. This cytokine promotes B cell proliferation and differentiation towards plasma cells [2]. Costimulation and IL-21 production by Tfh cells are also crucial for somatic hypermutation, affinity maturation and class switch recombination of B cells within germinal centers (GC) [3].

Tfh cell-mediated B cell help is naturally regulated by T follicular regulatory (Tfr) cells. Tfr cells are a specialized subset of regulatory T (Treg) cells and are able to suppress both Tfh cells and B cells within the GC [4–6]. In particular the ratio between Tfh cells and Tfr cells seems to control the magnitude of the antibody response [7]. An important mode of suppression by Treg cells is through the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). This inhibitory receptor is even more highly expressed on Tfr cells [5,8,9]. Possible mechanisms of suppression by Treg cells through CTLA-4 are I) physical sequestering of CD80/86 on antigen-presenting cells (APC), hereby attenuating CD28 signaling and T cell activation, II) transendocytosis of CD80/86 ligands upon interaction with APCs, and III) intrinsic competition with CD28 for CD80/86 binding at the immune synapse [10].

While several studies have shown that proportions of circulating Tfh (cTfh) cells are increased in pSS patients compared with healthy controls (HCs) [11–15], the prevalence of cTfr cells in pSS is rather unexplored. Circulating cTfh and cTfr cells are thought to function as a memory pool to enable fast and strong, but controlled responses upon antigen re-encounter, although the suppressive capacity of circulating Tfr (cTfr) cells seems lower compared to Tfr cells in the GC [16]. Changes in proportions of Tfr cells and expression of inhibitory receptors, such as CTLA-4, may affect Tfh cell formation and function, as well as B cell responses. The aim of this study was to assess the frequency and phenotype of cTfr cells and cTfh cells in pSS. Furthermore, the relation between cTfr and cTfh cell frequencies, B cell activity, and systemic disease activity were studied. We found that cTfr cells were expanded in pSS patients, even to a larger extent than cTfh cells. The frequency of cTfr cells correlated positively with serum levels of IgG and CXCL13, and with systemic disease activity. Finally, we observed that circulating Treg cells, and in particular cTfr cells, from pSS patients express decreased levels of CTLA-4.

PATIENTS AND METHODS

Study population

Twenty-four pSS patients (23 female, mean age 44 years) and 24 sex- and age-matched HCs (23 female, mean age 43 years) were included. All pSS patients participated in a previously reported, open-label re-treatment study with rituximab [17]. Twenty-four patients of this cohort were analyzed in the current study; 4 patients were not included because of unavailability of stored peripheral blood mononuclear cell (PBMC) samples (n=3) or serum sickness-like manifestations after the first dose of RTX (n=1). Patients were included at least one year after the last rituximab infusion, after reappearance of circulating B cells to baseline levels, and recurrence of symptoms. Only baseline samples before start of re-treatment were included in the current study. Characteristics of the study population have been described previously [14,18]. All patients retrospectively fulfilled 2016 ACR-EULAR criteria for pSS [19]. In addition, samples of 44 recently diagnosed, biologic treatment-naive pSS patients, included in an inception cohort, were analyzed (43 female, mean age 53 years). These patients prospectively fulfilled 2016 ACR-EULAR criteria for pSS.

For all patients included in the current study, systemic disease activity was prospectively assessed by the ESSDAI and Clinical ESSDAI (ESSDAI without inclusion of the biological domain) [20,21]. All patients and HCs provided written informed consent. The study was approved by the Medical Ethics Committee of the University Medical Center Groningen (METc2008.179/METc2013.066).

Flow cytometry analysis

Cryopreserved PBMC samples were thawed and of each sample two million cells were fluorescently labeled for immunophenotyping of $CD4^+$ T cells by flow cytometry. A previously reported panel of antibodies was used [14], with addition of anti-human CTLA-4-BV421 (clone BNI3, BD Biosciences) for the 24 baseline samples of the rituximab cohort and HCs. Both membrane-bound and intracellular CTLA-4 expression was measured, because it is highly endocytic, resulting in approximately 90% of CTLA-4 being intracellular [10]. The Foxp3 transcription factor fixation/permeabilization concentrate and diluent solutions (eBioscience) were used for staining of CTLA-4 (and FoxP3).

Circulating Tfh cells were defined as $CD4^+CD45RA^-FoxP3^-CXCR5^+PD\text{-}1^+$, cTfr cells as $CD4^+CD45RA^-FoxP3^+CXCR5^+PD\text{-}1^+$ and peripheral Treg (pTreg) cells as $CD4^+CD45RA^-FoxP3^+CXCR5^-$ cells. Median expression levels per cell of CTLA-4 in cTfr cells and pTreg cells were measured by median fluorescence intensity (MFI). Flow cytometric measurements were performed on a FACS-LSRII flow cytometer (Becton Dickinson) and data were analyzed using FlowJo software (Tree Star).

Analysis of CXCL13 levels in serum

In patients from the inception cohort, serum levels of CXCL13 were measured by an enzyme-linked immunosorbent assay (ELISA) using the DuoSet ELISA Human CXCL13 development system (R&D systems), according to the manufacturer's protocol.

Statistical analysis

Statistical analyses for comparisons between pSS patients and HCs were performed using Mann–Whitney U test. Correlations were evaluated with Spearman's Rho tests. Two-tailed P values <0.05 were considered statistically significant.

RESULTS

Frequency and phenotype of cTfh cells and cTfr cells in pSS

Immunophenotyping of $CD4^+$ T cells revealed that pSS patients presented with higher frequencies of circulating cTfh cells, as well as cTfr cells, compared with HCs (Figure 1A and 1B; P<0.001). Circulating Tfr cells were relatively further increased than cTfh cells, resulting in significantly higher cTfr/cTfh ratios in pSS patients, compared with HCs (Figure 1C; P<0.001). Frequencies of cTfh cells and cTfr cells were comparable between pSS patients from the rituximab cohort and the inception cohort, indicating that treatment with rituximab in the past had no effect on these frequencies.

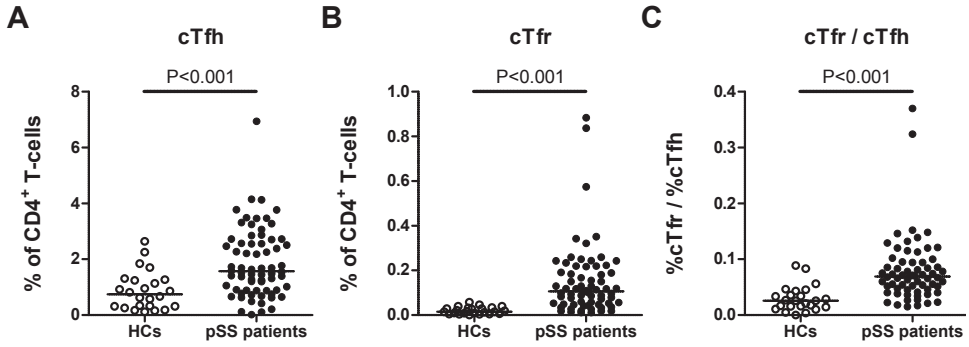

FIGURE 1 | Frequencies of cTfh cells, cTfr cells and the cTfr/cTfh ratio in pSS patients and healthy controls. Within the $CD4^+$ T-cell compartment, frequencies of (A) cTfh cells ($CD45RA^-FoxP3^-CXCR5^+PD\text{-}1^+$) and (B) cTfr cells ($CD4^+CD45RA^-FoxP3^+CXCR5^+PD\text{-}1^+$) were assessed and the cTfr/cTfh ratio (C) was calculated. Baseline data from pSS patients in the rituximab group (n=24) and data from the inception cohort (n=44) were combined. HC: healthy control. Horizontal lines indicate the median. P-values were calculated using the nonparametric Mann-Whitney U-test.

To investigate the migratory potential of circulating cTfh cells and cTfr cells to inflamed tissue in pSS, expression of CXCR3 and CCR6 was measured. We found larger proportions of CXCR3-expressing cTfh and cTfr cells in pSS patients compared with HCs (Figure 2). The $CXCR3^{+}CCR6^{-}$ phenotype, similar to Th1 cells [22], was predominant in pSS, both for cTfh cells (median 67%) and cTfr cells (median 44%). Frequencies of cTfh and cTfr cells with a $CXCR3^{-}CCR6^{+}$ phenotype, similar to Th17 cells, were not significantly different between pSS patients and HCs (Figure 2).

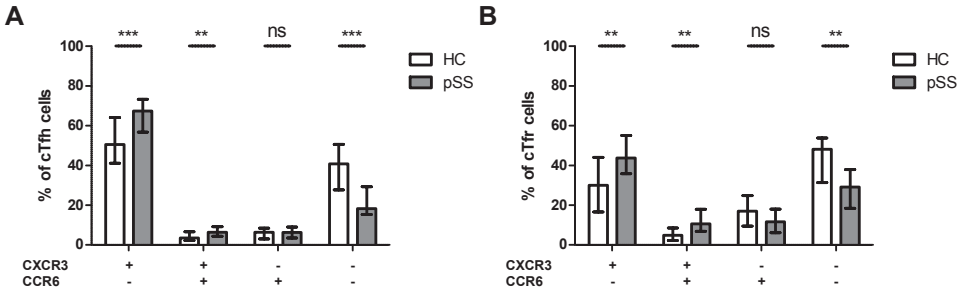

FIGURE 2 | Chemokine receptor expression by cTfh and cTfr cells. Expression of CXCR3 and CCR6 by circulating T follicular helper (cTfh) cells (A) and circulating T follicular regulatory (cTfr) cells (B) was measured in 24 pSS patients and 24 age- and sex-matched HCs. Bars indicate the median and interquartile range. P-values were calculated using the nonparametric Mann-Whitney U-test. *** P<0.001; ** P<0.01; ns: not significant (P≥0.05); HC: healthy control.

Correlations between cTfh and cTfr cell frequencies, B cell hyperactivity and disease activity

Next, we assessed whether cTfh or cTfr cell frequencies were associated with serological markers of B cell hyperactivity and systemic disease activity in pSS patients. Both cTfh and cTfr cell frequencies correlated significantly with serum levels of IgG and immunoglobulin free light chains (Figure 3 and data not shown). Frequencies of both cell subsets also correlated significantly with serum levels of CXCL13 (Figure 3). CXCL13 is an important chemokine for B cell homing to lymphoid follicles and is involved in ectopic lymphoid tissue formation in pSS [23,24]. On the other hand, the serum level of CXCL10, a biomarker that is associated with pSS[25], but not directly with B cell activity, was not significantly associated with cTfh or cTfr cell frequencies (data not shown). Together, these results indicate that increased frequencies of cTfh and cTfr cells are related to T cell-dependent B cell activity in pSS. Furthermore, these cell subsets correlated significantly with systemic disease activity, as measured by ESSDAI and clinESSDAI scores. (Figure 3).

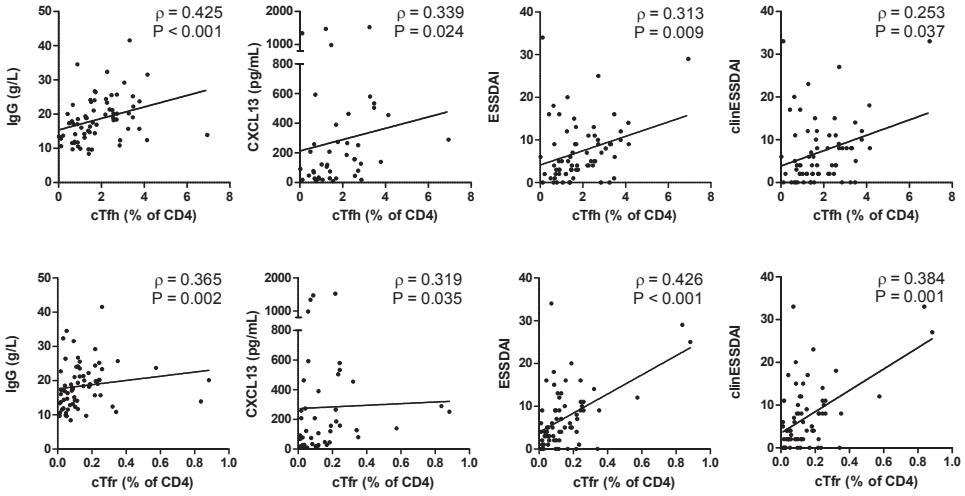

FIGURE 3 | Correlations between cTfh and cTfr cell frequencies, signs of B cell hyperactivity and disease activity in pSS. Frequencies of (A) circulating T follicular helper (cTfh) cells and (B) circulating T follicular regulatory (cTfr) cells were correlated with serum levels of IgG and CXCL13, and ESSDAI scores. Baseline data from pSS patients in the rituximab group (n=24) and data from the inception cohort (n=44) were combined. Correlations were evaluated with Spearman's correlation coefficient (ρ). ESSDAI: EULAR Sjögren's syndrome disease activity index.

CTLA-4 expression by cTfr cells and other regulatory T cells in pSS

In addition to markers for phenotypic classification of human $CD4^+$ T cells, median expression levels (median fluorescence intensity; MFI) of CTLA-4 were assessed. CTLA-4 is important for immune suppression and alterations in CTLA-4 expression may affect immune homeostasis in pSS. We observed that levels of CTLA-4 in peripherally induced Treg (pTreg) cells as well as cTfr cells were significantly lower in pSS patients, compared with HCs (Figure 4C). The highest difference in CTLA-4 expression between pSS patients and HCs was seen in activated cTfr cells ($CXCR5^+PD\text{-}1^+FoxP3^{high}$). These data suggest that the immune suppressive potential of pTreg cells, and in particular cTfr cells, is reduced in pSS patients. Since Tfr cells negatively regulate formation of Tfh cells [8], we correlated the MFI of CTLA-4 in cTfr cells to the frequency of cTfh cells. In patients with lower CTLA-4 expression levels, the frequency of cTfh cells tended to be higher (Figure 4D). The correlation between CTLA-4 expression levels by activated ($FoxP3^{hi}$) cTfr cells and frequencies of cTfh cells was even stronger (Spearman's ρ=0.60, P=0.002). Together, these findings indicate that the suppressive capacity of cTfr cells is possibly reduced in pSS patients with decreased CTLA-4 expression.

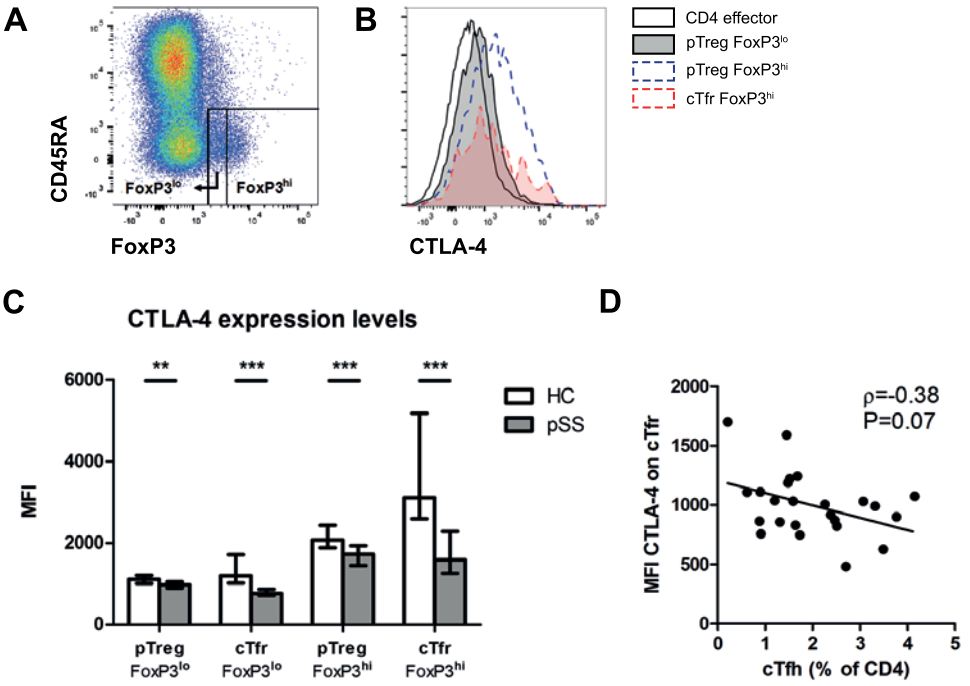

FIGURE 4 | Altered expression of CTLA-4 by regulatory T cells in pSS. (A) Representative dot plot of CD45RA and FoxP3 expression in $CD3^+CD4^+$ cells from a pSS patient. Regulatory cells were subdivided into $FoxP3^{lo}$ (resting) and $FoxP3^{hi}$ (activated). (B) Representative histogram of CTLA-4 expression levels in different $CD4^+$ cell subsets from a pSS patient. (C) Median fluorescence intensity (MFI) of CTLA-4 in resting and activated circulating pTreg cells ($CD45RA^-FoxP3^+CXCR5^-$) and circulating T follicular regulatory (cTfr) cells ($CD45RA^-FoxP3^+CXCR5^+PD\text{-}1^+$). Data from 24 pSS patients and 24 healthy controls (HC) are displayed. P-values were calculated using the nonparametric Mann-Whitney U-test. Bars indicate the median and interquartile range. (D) Correlation between CTLA-4 expression levels by cTfr cells and frequencies of circulating T follicular helper (cTfh) cells, evaluated with Spearman's correlation coefficient (ρ). *** P<0.001; ** P<0.01.

DISCUSSION

Tfh cells are elevated in the inflamed salivary glands and peripheral blood of pSS patients and are likely involved in the establishment of T cell-dependent B cell hyperactivity [14,15,26,27]. Tfr cells are important regulators of Tfh cell proliferation and B cell activation [28]. This study shows that not only cTfh, but also cTfr cell frequencies are increased in pSS patients compared with HCs, even to a larger extent than cTfh cells. A major proportion of cTfr cells in pSS patients expressed CXCR3, promoting CXCL10-driven migration to inflamed tissues. Frequencies of cTfr cells correlated positively with serological markers of B cell activity and with systemic disease activity (i.e., ESSDAI and clinESSDAI scores). The increased frequencies of cTfr cells coincided with reduced expression of CTLA-4 by these cells, indicating that their suppressive potential is affected.

Recently, Fonseca et al. also showed that cTfr cells were increased in pSS patients [27,29]. In contrast to our study, they defined cTfr cells as $CD4^+CXCR5^+Foxp3^+$ T cells, comprising both naive and memory T cells. They showed that the majority of cTfr cells were $CD45RA^+Foxp3^{lo}$ resting, naive-like Treg cells [29]. These cells were able to suppress effector T cell proliferation, but lacked full B cell-suppressive capacity [29]. The authors suggested that the increase in cTfr cells could be a result of ongoing T cell activation in secondary lymphoid organs, leading to an increased output of cTfr (and cTfh) cells. In our study, cTfr cells were defined as $CD4^+CD45RA^-FoxP3^+CXCR5^+PD\text{-}1^+$ T-cells, hereby selecting the smaller proportion of antigen-experienced, memory-like cells. In mice, these memory-like cTfr cells were shown to persist in the circulation for (at least) 30 days, with the capacity to home to GCs after T cell reactivation [16]. These memory-like cTfr cells are therefore likely also the cells that migrate to the inflamed glandular tissues. As a result of a greater rise in cTfr cells compared with cTfh cells, the cTfr/cTfh ratio is increased in pSS patients. In B cell follicles, a higher Tfr/Tfh ratio negatively regulates antibody responses [30]. However, immune responses are clearly not sufficiently suppressed in pSS. CTLA-4 is an important receptor for immune suppression by Treg cells. We found that expression levels of CTLA-4 are decreased in circulating pTreg cells, including cTfr cells, from pSS patients compared with HCs. As CTLA-4 reduces availability of CD80/86 on APCs [8], lower CTLA-4 levels may result in less efficient suppression of Tfh cell expansion and consequently increased B cell activity. Consistent with this notion, we observed a reverse correlation between cTfh cell frequencies and CTLA-4 expression levels by cTfr cells.

Lower expression of CTLA-4 by Treg cells may not only affect proliferation of effector cells, like Tfh cells, but also proliferation of the Treg population itself can be enhanced [31,32]. In line with this notion, in the experimental autoimmune encephalomyelitis (EAE) mouse model, CTLA-4 deletion in Treg cells from adult mice resulted in expansion of effector T cells as well as Treg cells. Interestingly, we observed previously that frequencies of pTreg cells are also increased in peripheral blood of pSS patients [15]. The work presented here suggest that lower CTLA-4 levels may contribute to this expanded pool of pTregs. The implications of higher pTreg and cTfr cell frequencies for immune suppression and disease activity in pSS still need to be elucidated. Furthermore, the origin of these cell subsets remains controversial. A recent study in mice showed that Tfr cells could be derived from either $FoxP3^+$ or $FoxP3^-$ precursor cells [33]. This study also showed that Tfr cells can be specific for self-antigen as well as foreign (immunized) antigen. However, another study in mice showed that Tfr cells were not specific for the immunized antigen, and instead expressed a TCR repertoire closely resembling that of thymus-derived natural Treg cells [34]. Because natural Treg cells have a TCR repertoire that is skewed towards self-antigen recognition [35], the latter study suggest that Tfr cells are important for the suppression of autoimmune responses. Whether the

expanded cTfr cell population in pSS patients recognizes mainly self-antigens or foreign antigens remains unknown and needs further investigation.

In summary, the expansion of cTfr cells in pSS is associated with increased B cell activity as well as systemic disease activity. Apparently, these cells do not exhibit (full) suppressive function and fail to control B cell responses once they are recruited to secondary lymphoid organs and/or ectopic lymphoid tissue in the inflamed glands. Although lower CTLA-4 expression is suggestive of lower suppressive potential, the functional capacity of memory-like cTfr cells needs to be investigated to confirm this assumption. Novel insights into the role of cTfr cells in pSS pathogenesis may lead to development of new therapies to enhance or mimic suppressive function of these cells and attenuate B cell activity. In addition, therapies that reduce the elevated levels of cTfh and pTreg cells in pSS patients, as we have shown for abatacept [15], may (partially) restore the disturbed immune homeostasis in this disease.

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3B

IS THE T FOLLICULAR REGULATORY / T FOLLICULAR HELPER CELL RATIO IN BLOOD A BIOMARKER FOR ECTOPIC LYMPHOID STRUCTURE FORMATION IN SJÖGREN'S SYNDROME?

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Comment on 'Blood T Follicular Regulatory Cells / T Follicular Helper Cells ratio Marks Ectopic Lymphoid Structure Formation and PD-1+ ICOS+ T Follicular Helper Cells Indicate Disease Activity in Primary Sjögren's Syndrome' by Fonseca et al. (2018)

Arthritis Rheumatol. 2018 (in press)

We read with great interest the article by Fonseca et al [1], that was published in a recent issue of Arthritis & Rheumatology. The authors elegantly showed that T follicular regulatory (Tfr) cells were enriched in blood as well as in matched minor salivary gland (MSG) biopsies from patients with primary Sjögren's syndrome (pSS). They also showed that the Tfr/Tfh ratio in blood was increased in pSS compared to non-SS sicca patients. Interestingly, this Tfr/Tfh ratio in blood correlated with ectopic lymphoid structure formation in MSG tissue. To our opinion the authors did, however, not show a direct correlation between aberrant Tfr/Tfh ratios and ectopic lymphoid structure formation among pSS patients. In essence their study showed that in pSS patients the Tfr/Tfh ratio in blood was correlated with numbers of infiltrating lymphocytes, as assessed by flow cytometric analysis of MSG cell suspensions. Additionally, the authors showed that the Tfr/Tfh ratio in blood was increased in patients with focal sialoadenitis (FSA) (defined in their study as a focus score ≥1), compared to patients without FSA. Of note, this comparison was made irrespective of a diagnosis of pSS, which implicated that the majority of patients without FSA were non-SS sicca patients.

We assessed the number of circulating Tfr cells and Tfh cells in a larger inception cohort of 98 sicca patients clinically suspected of pSS. MSG biopsies of all patients were assessed in detail by histopathological analysis. Forty-four patients were classified as pSS (43 females, mean age 53, mean ESSDAI 7), and 54 patients as non-SS sicca patients (46 females, mean age 48). Of the 44 pSS patients, 80% were naive for treatment with corticosteroids or disease-modifying anti-rheumatic drugs. Consistent with the findings by Fonseca et al [1], frequencies of Tfr cells and the Tfr/Tfh ratio in blood were significantly increased in pSS compared to non-SS sicca patients (Figure 1A). In contrast to what has been suggested by Fonseca et al [1], we could not demonstrate in this larger inception cohort that pSS patients with a focus score ≥1 in MSG tissue had a higher Tfr/Tfh ratio in blood than pSS patients with a focus score <1. (Figure 1A). Moreover, neither focus score nor area of the $CD45^+$ infiltrate was correlated with the blood Tfr/Tfh ratio (Figure 1B). The Tfr/Tfh ratio was also not associated with ultrasonographic score of the major salivary glands (sUS) (Spearman's ρ=-0.04, P=0.831), while sUS was significantly associated with focus scores in both labial and parotid gland biopsies [2]. Thus, although our data also show that pSS patients have higher Tfr/Tfh ratios in blood, we found no association between this ratio in blood and glandular inflammation.

Besides increased levels of Tfr cells and the Tfr/Tfh ratio in blood, we also observed a significant increase in the frequency of activated ($PD\text{-}1^+ICOS^+$) Tfh cells in pSS compared to non-SS sicca patients (Figure 1C), while Fonseca et al. only observed a tendency towards higher frequencies of activated Tfh cells. Nonetheless, similar to the observations of Fonseca et al [1], we found that frequencies of activated Tfh cells in blood were associated with ESSDAI scores in pSS patients (Figure 1D). In addition, we observed that frequencies of activated Tfh cells correlated with Clinical ESSDAI

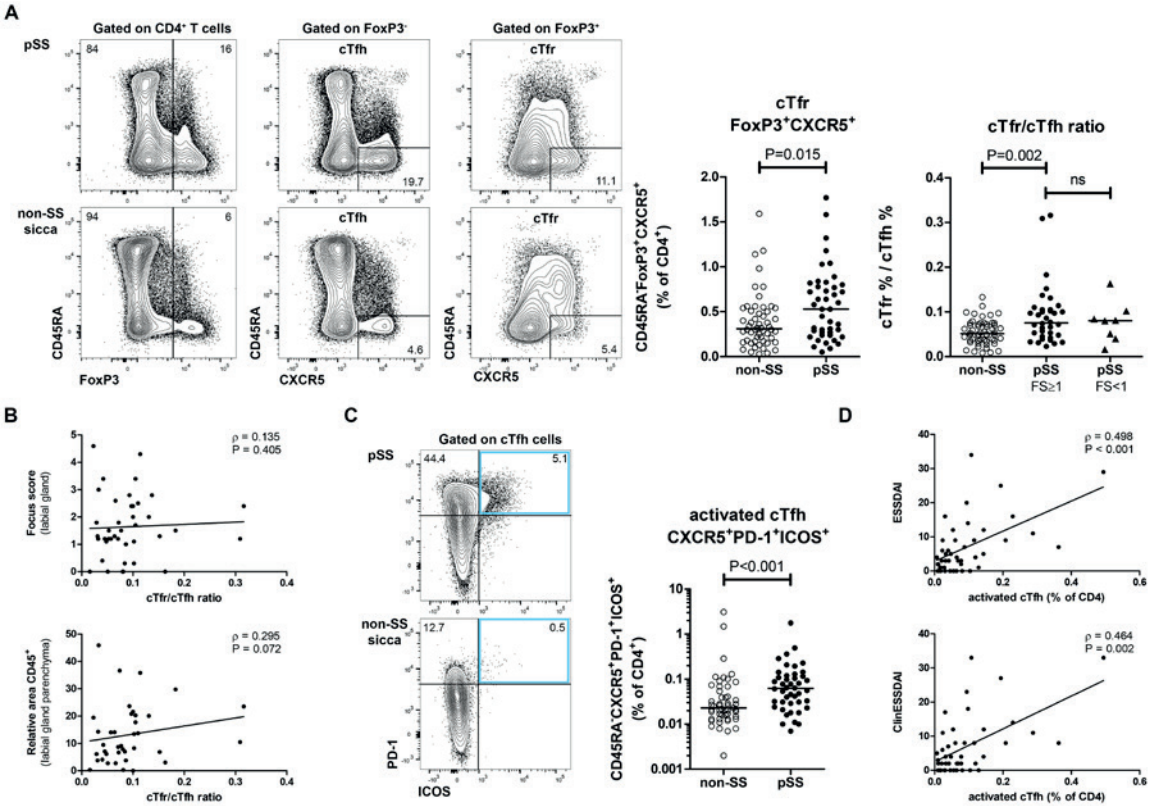

FIGURE 1. A, Gating strategy for circulating T follicular helper (cTfh) cells and T follicular regulatory (cTfr) cells, frequencies of cTfr cells, and the cTfr/cTfh ratio in 54 non-SS sicca patients, 36 pSS patients with FS≥1 and 8 pSS patients with FS<1. **B**, Correlations between the cTfr/cTfh ratio, FS in labial gland sections (n=40), and the relative area of parenchyma positively stained for CD45 in labial gland sections (n=38), in pSS patients. **C**, Gating strategy for activated Tfh cells, and frequencies of this cell subset in blood from 54 non-SS sicca and 44 pSS patients. Light blue box shows gate for activated cTfh cells. **D**, Correlations between activated Tfh cell frequencies, ESSDAI scores, and ClinESSDAI scores in blood from 44 pSS patients. Horizontal lines indicate the median. Circles indicate individual subjects. FS = focus score (number of foci per 4 mm^2 parenchyma); ρ = Spearman's Rho correlation coefficient; ESSDAI = EULAR Sjögren's syndrome disease activity index.

(ESSDAI without the biological domain [3]) scores (Figure 1C), indicating that the correlation is not only based on activity in the biological domain (e.g., hypergammaglobulinemia). Support for an association between activated Tfh cells and disease activity also comes from our previous study, in which circulating Tfh cells in pSS patients were studied before and after treatment with abatacept [4]. In that study we observed a significant decrease in activated Tfh cells in blood during treatment. Furthermore, the reduction of ICOS expression by the remaining Tfh cells correlated significantly with the decrease in ESSDAI scores [4].

In conclusion, the data presented by Fonseca et al. provides evidence that Tfr and Tfh cells are important players in pSS pathogenesis [1]. Likely, these cells are involved in B cell hyperactivation that characterizes this disease, but levels of these cells in blood may not necessarily reflect the presence of ectopic lymphoid tissue in the salivary glands. Importantly, all available data do indicate that Tfh cells contribute significantly to systemic disease activity in pSS, and emphasize that these cells are an important target for treatment.

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4

GENE EXPRESSION PROFILING OF EPITHELIUM-ASSOCIATED FCRL4^{+} B CELLS IN PRIMARY SJÖGREN'S SYNDROME REVEALS A PATHOGENIC SIGNATURE

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Work in progress

ABSTRACT

Objective

A small subset of B cells in blood expresses Fc-receptor-like protein 4 (FcRL4). In healthy individuals, FcRL4 expression is mostly limited to B cells in mucosal tissues. In patients with primary Sjögren's syndrome (pSS), $FcRL4^+$ B cells are found in inflamed salivary gland tissue, in particular within parotid glands. At these sites, $FcRL4^+$ cells reside in close proximity to ductal epithelial cells. FcRL4 is also expressed by mucosa-associated lymphoid tissue (MALT) lymphoma B cells. We aimed to assess the phenotype and frequency of circulating $FcRL4^+$ B cells as well as the transcriptional profile of $FcRL4^+$ B cells from parotid glands of pSS patients.

Methods

The frequency and phenotype of $FcRL4^+$ B cells in blood was assessed by flow cytometry. Peripheral blood mononuclear cells of 44 pSS patients and 54 non-SS sicca patients were included. Additionally, fresh parotid gland biopsies from 6 pSS patients were obtained. Of these biopsies cell suspensions were prepared, which were incubated with appropriate antibodies and sorted as single-cells or 5 cells per well ('bulk') based on the following definitions: $CD19^+CD27^-FcRL4^-$ ('naive') $CD19^+CD27^+FcRL4^-$ ('memory') and $CD19^+FcRL4^+$. Preparation of cDNA libraries was done using an in-house SMARTseq2 protocol and sequencing was performed on an Illumina NextSeq500.

Results

Frequencies of circulating $FcRL4^+$ B cells were not significantly altered in pSS patients compared with non-SS sicca controls. The majority of $FcRL4^+$ B cells in pSS patients and controls had a $CD21^{lo}CXCR3^+$ phenotype. For RNA sequencing analysis of glandular $FcRL4^+$ B cells, samples from 5 out of 6 pSS patients passed quality control. Of these 5 patients, one was diagnosed with MALT lymphoma and therefore excluded from differential expression analysis. From the remaining patient samples a total of 150 single B cells and 360 B cells in 'bulk' were included in the analysis. Both in single-cell and 'bulk' analysis, multiple genes coding for integrins, such as *ITGAX* (CD11c), were significantly upregulated in $FcRL4^+$ B cells. Gene Ontology pathways that showed the highest upregulation in $FcRL4^+$ B cells (both single-cell and 'bulk') were receptor binding, GTPase and protein kinase pathways. In 'bulk' samples genes encoding for Src tyrosine kinases, genes involved in the NF-κB pathway, *CXCR3*, and *TNFRSF13B* (TACI) were significantly upregulated in $FcRL4^+$ B cells, compared with either $CD27^-FcRL4^-$ or $CD27^+FcRL4^-$ B cells. Gene expression levels of *CD40* and *LCK* were significantly decreased in $FcRL4^+$ B cells.

Conclusion

Circulating FcRL4$^+$ B cells are mostly activated cells, reflected by a CD21^{lo}CXCR3$^+$ phenotype. The frequency of these cells was similar in pSS patients and non-SS sicca controls. FcRL4$^+$ B cells in salivary glands of pSS patients show upregulation of genes involved in homing and cell adhesion, consistent with their location close to the epithelium. FcRL4$^+$ B cells also show upregulation of genes that promote inflammation and B cell survival, possibly in a T cell-independent manner. These cells exhibit all characteristics of chronically activated CD11c$^+$ memory B cells. We postulate that these cells contribute significantly to the epithelial damage seen in the glandular tissue of pSS patients.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease that affects about 0.04% of the general population [1], and predominantly woman. Salivary and lacrimal glands are the main target of the disease. Patients usually present with symptoms of dry mouth, dry eyes, and fatigue. Mononuclear infiltration, mainly consisting of $CD4^+$ T cells and B cells, is a characteristic histopathological finding in pSS patients. Infiltrates are mostly found around the ductal epithelium, but lymphocytes can even be present within the epithelium where they may become part of lymphoepithelial lesions (LEL) [2].

LELs are composed of proliferative metaplastic epithelial cells and intraepithelial lymphocytes, mostly B cells. The vast majority of these intraepithelial B cells express the inhibitory IgA-binding Fc receptor-like protein 4 (FcRL4) [3,4]. In healthy individuals, $FcRL4^+$ B cells are restricted to mucosal tissues and mesenteric lymph nodes, where they participate in mucosal immune responses [5]. Interestingly, $FcRL4^+$ B cells have also been found in the synovium of patients with rheumatoid arthritis (RA), and here they produce high levels of RANKL, thereby exhibiting a pathogenic role in this disease [6,7]. Analysis of immunoglobulin genes from $FcRL4^+$ B cells in RA showed high levels of hypermutation in the variable regions [6], which indicates involvement of antigen and T cells in the selection of these cells. Synovial $FcRL4^+$ B cells from RA patients harbored autoreactive clones and mostly used the IgG1 and IgA1 isotypes. Usage of the IgA isotype was more frequent in $FcRL4^+$ compared with $FcRL4^-$ B cells [6]. These studies in RA patients indicate that $FcRL4^+$ B cells are activated cells that may play a major role in local immune responses in autoimmune diseases, and that these cells can contribute to tissue damage.

Patients with pSS have a significantly increased risk for development of non-Hodgkin lymphomas, mostly of the mucosa-associated lymphoid tissue (MALT) type [8]. MALT lymphomas in pSS develop preferentially in the parotid glands. The neoplastic B cells of parotid MALT lymphomas are located in and around LELs, and widely express FcRL4 [9]. Hence, we postulated that intraepithelial FcRL4-expressing B cells may be the cells from which MALT lymphomas in parotid glands arise.

Whether glandular $FcRL4^+$ B cells have been activated at mucosal tissue sites (e.g., gut mucosa) and subsequently migrate to the inflamed salivary glands of pSS patients, or whether they are formed locally in these glands is not known. Also their functional properties within the inflamed salivary glands are not clear. For these reasons we studied the phenotype and frequency of circulating $FcRL4^+$ B cells in pSS patients by flow cytometry, and the transcriptome of local $FcRL4^+$ B cells by single cell RNA sequencing, to gain insight in the phenotype and functional capabilities of these cells. We hypothesized that $FcRL4^+$ B cells migrate into in the inflamed glands of pSS patients

and contribute to pathogenesis by participating in LEL formation and by secretion of pro-inflammatory factors. Furthermore, we hypothesized that FcRL4+ B cells express genes that may predispose these cells to lymphomagenesis.

PATIENTS AND METHODS

Immunophenotyping of circulating B cells

Consecutive patients, referred to the Sjögren Expertise Center of the University Medical Center Groningen (UMCG) for suspicion of SS were included (*n*=98). Informed consent was obtained from all patients according to the Declaration of Helsinki and the study was approved by the Medical Research Ethics Committee of the UMCG (METc2013.066). Patients that fulfilled 2016 ACR-EULAR criteria for pSS were classified as pSS patients [10]. From the 98 patients included in our cohort, 44 patients were classified as pSS and 54 as non-SS sicca patients. All pSS patients were naive for treatment with biologic disease-modifying anti-rheumatic drugs. Two pSS patients were diagnosed with MALT lymphoma. Cryopreserved peripheral blood mononuclear cells were thawed and analyzed by flow cytometry for the presence of circulating FcRL4+ B cells. The following antibodies were used: anti-human-CD19-BV786 (clone SJ25C1), anti-human-CD27-BV421 (clone M-T271), anti-human-IgD-BUV395 (clone IA6-2), anti-human-CD21-BUV737 (clone B-ly4), anti-human-CXCR3-PE-Cy7 (clone 1C6), all from BD Biosciences, and anti-human-FcRL4-PE (clone 413D12, Biolegend). Fixable viability dye eF506 (eBioscience) was used for live/dead discrimination. Data were acquired on a FACS-LSRII flow cytometer (Becton Dickinson, USA) and analyzed using FlowJo software (Tree Star, USA).

Tissue samples for RNA sequencing

Parotid gland tissue was obtained fresh from 6 adult patients who were anti-SSA positive, had a high clinical suspicion of pSS, and underwent a diagnostic biopsy. All surgeries were performed at the department of Oral and Maxillofacial Surgery of the University Medical Center Groningen (UMCG), the Netherlands. Permission to collect these tissues for research purposes was obtained from the Medical Research Ethics Committee of the UMCG (METc2016/010).

Preparation of cell suspensions for RNA sequencing

Cell suspensions were prepared as described by Pringle et al. [11], with the following adaptions: Biopsies were manually cut using scissors, and the incubation period for enzyme-based digestion was 30 minutes. 32,5 µL digestion buffer was used per milligram of tissue. Cells were resuspended in MACS buffer (PBS/0.5% BSA/2 mM EDTA) for cell surface staining.

Flow cytometry analysis and sorting for RNA sequencing

Cell suspensions prepared from parotid gland tissue were incubated with antibodies (identified below) for 30 min at 4 °C, and washed twice in MACS buffer. The following antibodies were used: anti-human-CD19-eF450 (clone HIB19), anti –human-CD27-APC (clone O323), both from eBioscience, and anti-human-FcRL4-PE (clone 413D12, Biolegend). Immediately before sorting, cells were stained with Propidium Iodide (eBioscience) for live/dead discrimination. Gating was performed as described in supplementary figure 1. Cells were sorted by 1 or 5 cells/well into 96-well PCR plates containing 2 µl of lysis buffer (0.2% Triton X-100 (Sigma-Aldrich) + 2 U/µL RNAse inhibitor (Westburg-Clontech)), 1 µl of 10 µM oligo-dT_{30}VN primer (Biolegio) and 1 µl of 4 x 10mM dNTP mix (Westburg-Fermentas) per well. Cells were sorted on a MoFlo Astrios cell sorter (Beckman Coulter).

Preparation of cDNA libraries and sequencing

For cDNA library preparation the Smart-seq2 protocol by Picelli et al. was used [12,13], with the following adaptions: Reverse Transcriptase (RT) mastermix contained: 2.5 U SmartScribe RT, 0.25 U RNAse inhibitor (both from Westburg-Clontech), 1x SmartScribe first-strand buffer, 2 nM DTT (both from LifeTechnologies), 1 M Betaine (BioUltra ≥99.0%; Sigma-Aldrich), 1µM BC-TSO (Biolegio). After reverse transcriptase an exonuclease step was added to remove unbound oligo-dT primers. One µL of Exonuclease I (1:400 dilution in pure water) was added to each well and the plate was incubated 45 minutes at 37 °C, to activate the enzyme, immediately followed by 15 minutes at 85 °C to inactivate the enzyme. Samples were purified using Agencourt Ampure XP Beads (Beckman Coulter). The presence and size distribution of the obtained PCR product was measured on a PerkinElmer LabChip GX high-sensitivity DNA chip. Next, PCR products were pooled for tagmentation. Products from single cells were pooled 1:1, and equimolar pooling was performed for products from 5 cells/well. To tag the DNA with adapter sequences, a tagmentation step was performed using the Illumina Nextera XT DNA sample preparation kit, according to the manufacturer's protocol, with 500 pg of pooled cDNA. Subsequently, the subpools were indexed using a N7xx primer from the Nextera XT DNA sample preparation kit and a custom P5-TSO hybrid primer (10 uM) with the Nextera PCR mastermix. The concentration and size distribution of the obtained Nextera products were measured on a PerkinElmer LabChip GX high-sensitivity DNA chip and a superpool was prepared by equimolar pooling of the six Nextera products. The superpool was divided over four lanes and sequenced on a Illumina NextSeq500 instrument. The first read consisted of 18 bp, to sequence the cell-barcode (10 bp) and the UMI (7 bp), followed by the index read sequencing the Nextera index (8 bp). The second read consisted of the Nextera index plus 50 bp, sequencing the last part of the

captured gene. For single cells, the average sequencing depth was $4.2x10^6$ reads per cell.

Read alignment, quality control and gene expression estimation

Dropseq tools 1.12 was used to extract well barcodes and molecular barcodes (unique molecular identifiers; UMI's) from the reads [14]. The extracted well and UMI reads are flagged with BAM tags and then stored in BAM format together with the corresponding read. For quality control only reads with a well and UMI barcode with a minimum basecallQuality of 10 were included. Other reads were discarded. Of the remaining reads the SMART adapter and PolyA tails were removed using Dropseq. In the next step, reads were aligned to the human genome (hg19) using STAR v2.5.1b with default settings [15]. The aligned reads were filtered for uniquely mapping reads. Before gene quantification, aligned reads were sorted using Picard tools 2.2.2 (https://broadinstitute.github.io/picard). Ensembl version 75 was used to map protein-coding transcripts. For single cells, quantification of gene expression was performed using Dropseq filtering on unique UMI's.

Data analysis

All statistical analysis and plotting of RNA-seq data was performed using R software. For single cells, reads with unique molecular identifiers (UMI) were used as input, to correct for PCR duplicates [16]. Seurat version 2.1 was used for quality control, clustering and analysis of single cell data [17]. Single cells that expressed <200 genes and genes that were expressed in <3 cells overall were excluded. Also cells with a mitochondrial gene percentage higher than three times the median absolute deviation across all cells were excluded [18]. For differential expression analysis of single cell data, the MAST package was used [19]. For differential expression analysis of 'bulk' (5 cells/well) samples, DESeq2 was used [20], with 6 biological replicates per sample. Raw read counts (not corrected for UMI duplicates) were used as input, because DESeq2 expects un-normalized counts, and internally corrects for library size. P-values were adjusted by the Benjamini-Hochberg method and are hereafter indicated as false detection rate (FDR).

RESULTS

1. Frequency and phenotype of circulating FcRL4+ B cells in pSS patients and non-SS sicca patients

First, we compared the frequencies of circulating FcRL4+ B cells between pSS patients and non-SS sicca patients. Additionally, we measured co-expression of multiple B cell-related markers, including CD27, CD21, and CXCR3 (Figure 1A). We found no significant

difference in frequencies of circulating FcRL4$^+$ B cells between pSS patients and non-SS sicca patients (Figure 1B). Also absolute numbers of these cell subsets were not significantly altered (data not shown). Two pSS patients with MALT lymphoma did not show aberrant frequencies of these cells either. Circulating FcRL4$^+$ B cells comprised both CD27$^-$ 'naive' and CD27$^+$ 'memory' cells. A large proportion of circulating FcRL4$^+$ B cells expressed low levels of CD21 and co-expressed CXCR3 (Figure 1B). We did not observe significant phenotypical differences in FcRL4$^+$ B cells between pSS patients and non-SS sicca patients. Albeit their low prevalence, the phenotype of circulating FcRL4$^+$ B cells suggests that these cells have the capacity to migrate to inflamed tissue sites by expression of CXCR3.

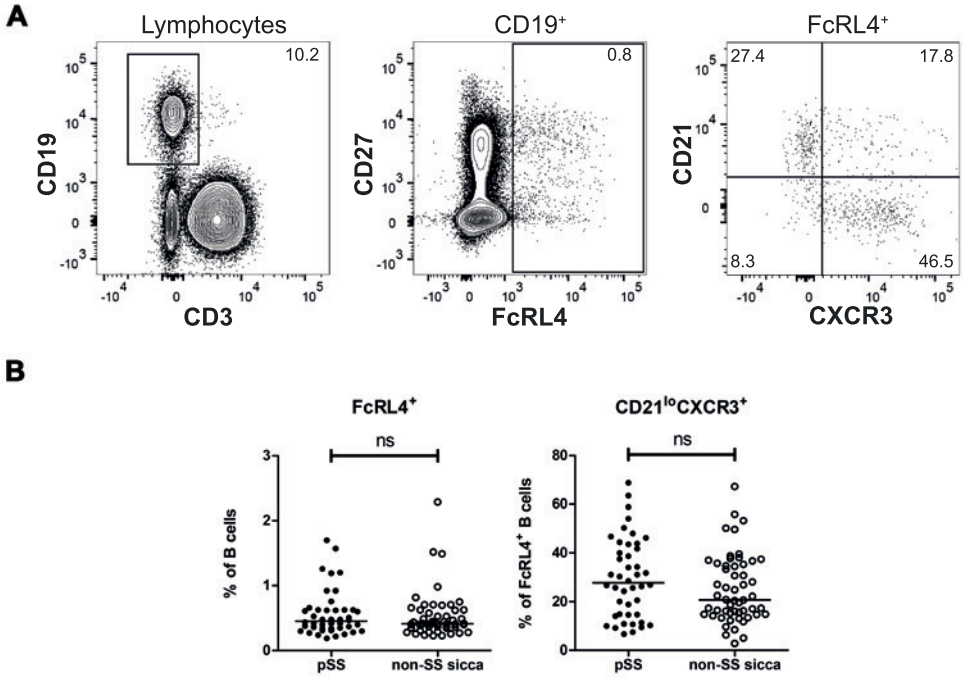

FIGURE 1 | FcRL4$^+$ B cells in peripheral blood of pSS patients and non-SS sicca patients. (A) Gating strategy used to identify FcRL4$^+$ B cells in peripheral blood. Lymphocytes were gated from single, live cells using forward and side scatter properties and fixable viability dye staining. (B) Frequencies of FcRL4$^+$ cells within the B cell compartment and frequencies of CD21^{lo}CXCR3$^+$ cells within the FcRL4$^+$ B cell compartment are shown. Data from pSS patients (*n*=44) and non-SS sicca patients (*n*=54) were included. P-value<0.05 was considered significant. Mann-Whitney U test was used for statistical analysis. Ns = not significant.

2. RNA sequencing of B cells isolated from salivary glands of pSS patients

To elucidate the phenotype and function of local FcRL4$^+$ B cells, we sorted these cells, as well as FcRL4$^-$ B cell subsets, from parotid gland tissues and isolated RNA for sequencing. Gene expression profiles were compared between FcRL4$^+$, FcRL4$^-$CD27$^-$ ('naive') and

FcRL4-CD27+ ('memory') B cells. Expression data of 'bulk' samples (5 cells/well) and single cells were analyzed separately. One patient (out of 6) was excluded before sequencing because of low RNA yields during library preparation for the 'bulk' samples. Single cells from this patient that were sequenced clustered highly distinct from other patients and were also excluded. Of the remaining 5 patients, one was diagnosed with MALT lymphoma. Data from this patient were excluded from differential expression analysis, because the transcriptional profile of these cells may bias the analysis of non-lymphoma B cells.

2.1 Differential expression analysis of 'bulk' cells.

Bulk cells (5 cells per subset per patient, 6 replicates) from all 4 patients were pooled in order to compare the gene expression profiles between subsets. In total 360 cells were included in the analysis. Gene Ontology pathway analysis using the PANTHER classification system[21,22] showed differential expression in receptor binding, ATPase, GTPase and protein kinase pathways in FcRL4+ B cells, compared with FcRL4-CD27- and FcRL4-CD27+ B cells. By using DESeq2 software to compare FcRL4+ B cells with FcRL4-CD27- ('naive') and FcRL4-CD27+ ('memory') B cells, we identified 1067 and 620 differentially expressed genes (FDR<0.05), respectively. Volcano plots in figures 2A and 2B illustrate the top differentially expressed genes based on significance level. The most distinctly expressed genes in FcRL4+ B cells, compared with FcRL4-CD27- or FcRL4-CD27+ B cells, were *FCRL4* (up) and *RGS16* (down). Next, we evaluated significantly differentially expressed genes with known immune function (Figure 2C). We found differential expression of several transcripts previously associated with FcRL4+ B cells [6,23]. These transcripts include the integrin *ITGAX* (CD11c), Src tyrosine kinases *HCK* and *FGR* (all up), *LCK* and *CXCR5* (down). We also found upregulation of multiple genes associated with activation of the NF-kappa B (NF-κB) signaling pathway: *NFKB1* (p50), *BCL2A1*, *MAP3K14* (NIK) and *TRAF3*, indicating that both canonical and non-canonical NF-κB pathways are active. Expression levels of *NFKBIA* (IκBα) and *NFKBID* (IκBNS), negative regulators of NF-kB, were significantly downregulated in FcRL4+ B cells. Other genes upregulated in FcRL4+ B cells were *THEMIS2, IL27RA, CD97, CXCR3, JAK2,* and *TNFRSF13B* (TACI). Downregulated genes included *MZB1* and *CD40*. Several genes with known immune function were only differentially expressed when comparing FcRL4+ B cells with FcRL4-CD27- ('"naive"') B cells. These genes include: *TGFBR1* , *IL18BP* , *IRF2*, *POLB*, *CD86*, *MYD88*, *TNF* and *SYK* (all up). These genes are apparently actively transcribed by both FcRL4+ B cells and FcRL4-CD27+ 'memory' B cells. A list of all differentially expressed genes with FDR<0.05 is available upon request from the corresponding author.

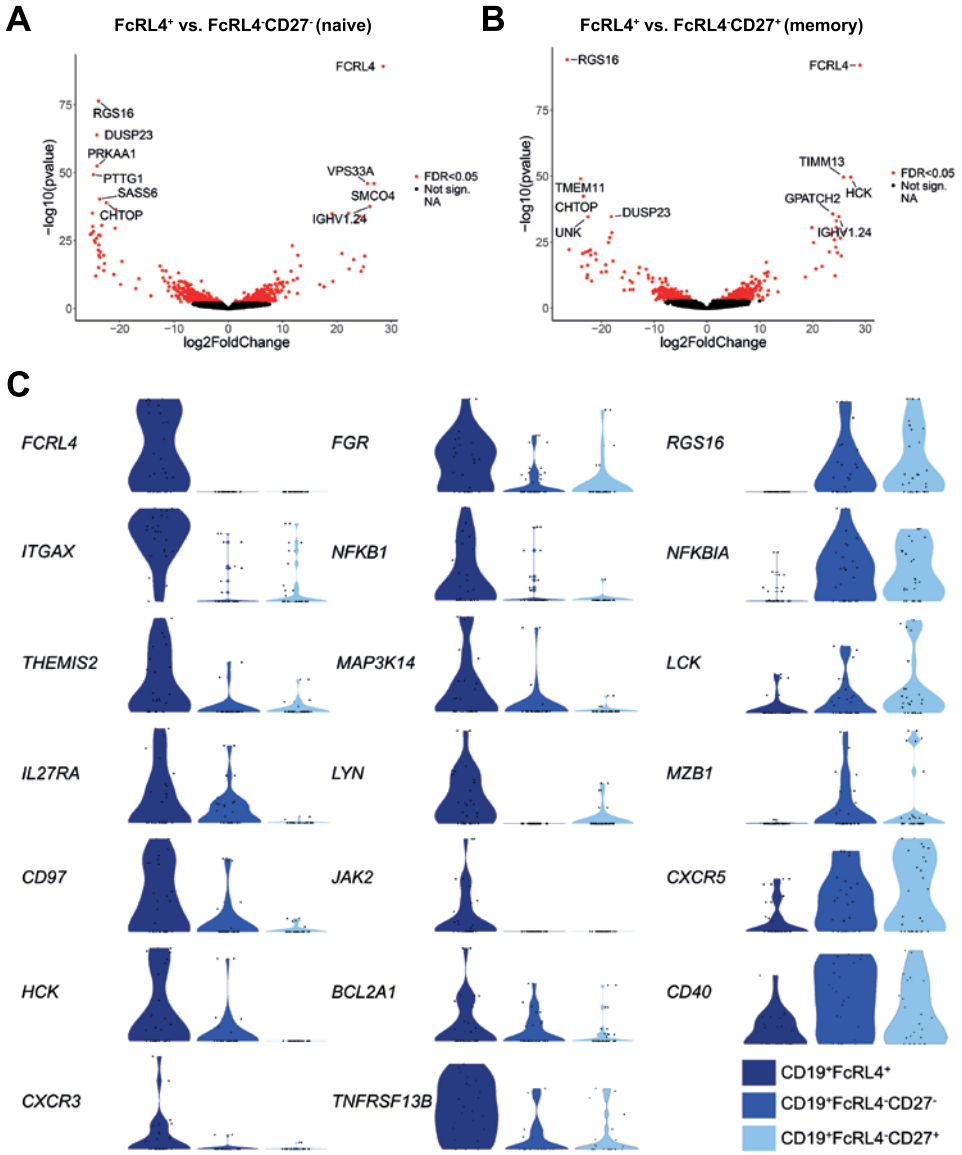

FIGURE 2 | Differential gene expression in FcRL4+ B cells at 'bulk' level. Differential gene expression for 5 cells/well samples was calculated using DESeq2 software. Volcano plots illustrate significantly differentially expressed genes (FDR<0.05, red) against non-significant genes (black). Differentially expressed genes in FcRL4+ B cells versus FcRL4-CD27- B cells (A), or versus FcRL4-CD27+ B cells (B) are displayed. In each volcano plot, ten genes with the lowest false detection rate (FDR) value are labeled. For each gene, the negative log10-transformed p-value is plotted on the y-axis and the log2-transformed fold change is plotted on the x-axis. A positive fold change indicates upregulated expression in FcRL4+ B cells. (C) Violin plots showing normalized counts (y-axis) per cell type (x-axis) for genes with known immune function that were significantly differentially expressed. Each black dot represents a 5-cell sample.

2.2 Differential expression analysis of single cells

Out of 159 sequenced parotid gland B cells, 9 cells were excluded after quality control using the Seurat package, mainly due to a high mitochondrial gene percentage. Thus, from 4 patients 150 single cells in total were included in the analysis. The lowest number of cells per B cell subset per patient was six, the highest 16 . To remove unwanted sources of variation in gene expression, data were scaled by regressing out the number of unique gene transcripts (the number of UMIs) per cell and the percentage of mitochondrial RNA per cell. Subsequently, unbiased clustering analysis was performed to assess if flow cytometry-defined cell subsets showed different gene expression patterns. When the first 12 principal components were projected onto two-dimensions by t-distributed stochastic neighbor embedding (t-SNE) [24], almost all flow cytometry-defined cell subsets per patient clustered separately (supplementary figure 2). In one patient, FcRL4$^+$ cells and FcRL4$^-$CD27$^+$ cells did not separate well based on tSNE cluster 1 and 2, which may indicate that in this patient the gene expression profile between FcRL4$^+$ cells and FcRL4$^-$CD27$^+$ cells is not highly distinct.

For differential expression analysis, we combined the various flow cytometry-defined cell subsets of all 4 patients. By using MAST software to compare FcRL4$^+$ B cells with FcRL4$^-$CD27$^-$ ('naive') and FcRL4$^-$CD27$^+$ ('memory') B cells, we identified 272 and 205 differentially expressed genes (FDR<0.05), respectively. Almost similar to the 'bulk' samples, Gene Ontology pathway analysis of single cells showed differential expression in receptor binding, nucleic acid binding, GTPase and protein kinase pathways in FcRL4$^+$ B cells, compared with FcRL4$^-$CD27$^-$ and FcRL4$^-$CD27$^+$ B cells. Volcano plots in figure 3A and 3B illustrate significantly differentially expressed genes against non-significant genes. *FCRL4*, *ITGAX*, *ITGB7*, *MLF2* and *TNFRSF13B* (TACI) were genes with known immune function that were significantly upregulated in FcRL4$^+$ B cells, compared with both FcRL4$^-$ subsets (Figure 3C). The following genes were differentially expressed in FcRL4$^+$, compared with FcRL4$^-$CD27$^-$ B cells: *ISG15*, *IRF7*, *OAS2*, *CD27*, *AICDA*, *SLAMF1* and *TGFBR2* (all upregulated). *BCLAF1* (transcriptional repressor of BCL2 family of proteins) and *PIK3IPI* (negative regulator of PI3K activity) were significantly downregulated. When comparing FcRL4$^+$ with FcRL4$^-$CD27$^+$ B cells, *CD84*, *IFI44*, *BCL2A1*, *PLCG2*, *CIITA*, *FGR*, *TNFSF10*, *TLR1*, and *CD48* were significantly upregulated. Expression levels of *IRF7* and *AICDA* were also upregulated, but not significantly (FDR=0.07 and 0.18, respectively). *CXCR4*, *CCR7* and *POLD2* were significantly downregulated. There were no genes with known immune function that were significantly downregulated in FcRL4$^+$ cells compared to both FcRL4$^-$ subsets. Although overlap in results is seen, several genes that were differentially expressed in the 'bulk' samples (figure 2C), were only rarely detected in the single cell samples.

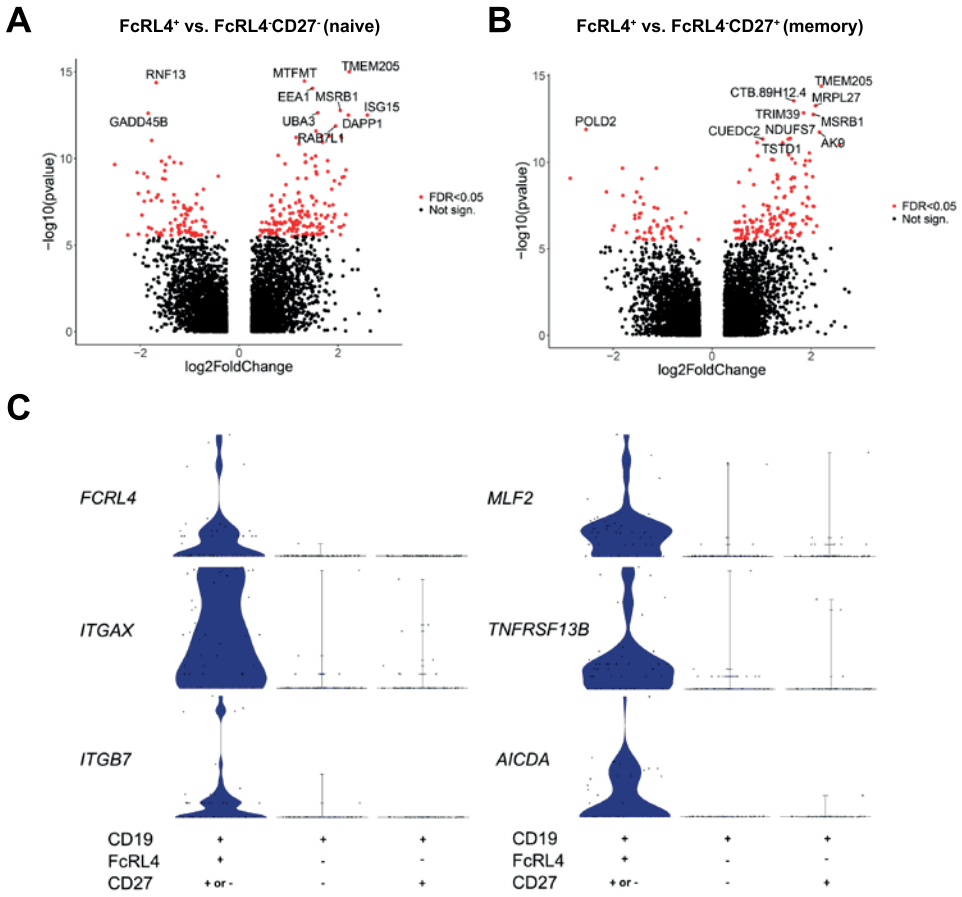

FIGURE 3 | Differential gene expression in FcRL4⁺ B cells at single cell level. Differential gene expression of single cells was calculated using MAST software. Volcano plots illustrate significantly differentially expressed genes (FDR<0.05, red) against non-significant genes (black) in FcRL4⁺ B cells versus FcRL4⁻CD27⁻ B cells (A), or versus FcRL4⁻CD27⁺ B cells (B). Log2FoldChange threshold was 0.25. Ten genes with the lowest false detection rate (FDR) value are labeled. For each gene, the negative log10-transformed p-value is plotted on the y-axis and the log2-transformed fold change is plotted on the x-axis. A positive fold change indicates upregulated expression in FcRL4⁺ B cells. (C) Violin plots showing log transformed expression (y-axis) per cell type (x-axis) for genes with known immune function that were significantly differentially expressed* when comparing FcRL4⁺ B cells with either FcRL4⁻CD27⁻ or FcRL4⁻CD27⁺ B cells. Each black dot represents a single cell sample. *FDR value of AICDA was >0.05 in the FcRL4⁻CD27⁺ comparison.

DISCUSSION

Recently, we showed that FcRL4 is expressed by intraepithelial B cells in the salivary glands of pSS patients. The origin, phenotype and functional capabilities of FcRL4⁺ B cells in the inflamed glandular tissue remain, however, poorly understood. Given their potential role in pSS pathogenesis, we assessed the frequency and phenotype of FcRL4⁺

B cells in the circulation of pSS patients, and investigated the transcriptional profile of FcRL4+ B cells located in the inflamed parotid glands. We observed that frequencies of *circulating* FcRL4+ B cells were generally low, and no difference in number or proportion of these cells was found between pSS patients and non-SS sicca patients. Albeit their low prevalence, we found that a large proportion of circulating FcRL4+ B cells expressed low levels of CD21 and co-expressed CXCR3, indicating that these cells are activated and programmed to migrate to inflamed tissues. In addition to immunophenotyping of circulating FcRL4+ B cells, we investigated, for the first time, the gene expression profile of *glandular* FcRL4+ B cells, isolated from parotid gland tissue of 4 pSS patients. We found multiple upregulated pathways that are involved in cell signaling, including receptor binding, GTPase and protein kinase pathways. Differentially expressed genes with known immune function could be subdivided into homing, B cell activation and lymphomagenesis pathways.

Homing

Gene expression analysis of the 'bulk' (5 cells) samples showed upregulation of CXCR3 in FcRL4+ B cells, together with downregulation of CXCR5. Similarly, flow cytometric analysis of circulating B cells showed that a large proportion of FcLR4+ B cells in blood co-expresses CXCR3. This may explain homing to the ductal epithelial cells of the salivary glands, which secrete high levels of the chemokine CXCL10/IP-10, the ligand for CXCR3. Additionally, integrins (e.g., *ITGAX* (CD11c)) and adhesion molecules (e.g, CD97) were upregulated in FcRL4+ B cells, compared with FcRL4- B cells. The expression of integrins and adhesion molecules by FcRL4+ B cells may result in retention of these cells around and within the epithelium by interaction with their ligands, such as ICAM-1. FcRL4+ B cells further exhibited increased transcript expression of Src family kinases (*HCK*, *FGR*, *LYN*), which are important for integrin signal transduction [25]. The upregulated expression of *ITGAX* (CD11c) and Src family kinases in glandular FcRL4+ B cells is consistent with results from earlier studies that analyzed the transcription profile of tonsillar FcRL4+ B cells or FcRL4+ B cells from synovia of patients with RA [6,23]. Upregulation of Src family kinases can contribute to antibody-induced inflammation, as mice that lack Src tyrosine kinases are protected from autoantibody-induced arthritis [26].

B cell activation

Previous studies have shown that enhanced expression of CD11c by memory B cells is associated with multiple autoimmune conditions and chronic immune stimulation [27]. CD11c+ memory cells are atypical memory cells, characterized by low expression of CD27 and CD21 [27]. These cells contain autoreactive specificities, are refractory to BCR stimulation, and respond robustly to TLR activation [27]. A similar pattern of

downregulated BCR signaling and enhanced TLR signaling is seen in FcRL4$^+$ B cells [28]. There is some evidence that binding of IgA to FcRL4 on the B cells is important for this switch from adaptive to innate signaling [28]. Negative regulation of BCR-induced signaling may be established by upregulation of *LYN,* as we observed in these cells. Lyn is a Src tyrosine kinase that can initiate, but also negatively regulate BCR signaling [29]. The transcriptional profile of FcRL4$^+$ B cells from the parotid glands further indicates that these cells have indeed been activated, possibly via TLR stimulation, as upregulation of multiple genes involved in both canonical and non-canonical NF-κB signaling was seen in the 'bulk' samples. Upregulation of genes involved in NF-κB signaling seems specific for FcRL4$^+$ B cells residing in the inflamed glandular tissue of pSS patients, since this was not reported for tonsillar FcRL4$^+$ B cells or synovial FcRL4$^+$ B cells [6,23]. Unexpectedly, the upregulation of NF-κB pathway genes in glandular FcRL4$^+$ B cells was not accompanied by increased expression levels of genes encoding for pro-inflammatory cytokines such as TNFα or IL-6. FcRL4$^+$ B cells did express higher levels of *TNF* compared to FcRL4-negative naive B cells, but not compared to FcRL4-negative memory B cells. Thus, effector functions of FcRL4$^+$ B cells that may contribute to epithelial damage and formation of LELs remain unclear. Another gene that was upregulated in FcRL4$^+$ B cells and that is involved in B cell activation and survival is TACI. Binding of BAFF/Blys or APRIL to TACI can enhance NF-κB signaling and promote B cell survival [30,31]. These cytokines are significantly upregulated in the salivary glands of pSS patients and are also produced by the ductal epithelial cells [32]. Binding of BAFF and/or APRIL to TACI expressed by FcRL4$^+$ B cells may promote their activation and survival. A less well-known gene involved in B cell activation that was also significantly upregulated in FcRL4$^+$ B cells was *THEMIS2*. Recently it was shown that THEMIS2 reduces the threshold for B cell activation by low-avidity antigens such as soluble proteins [33], which is clearly unfavorable under autoimmune conditions. Interestingly, this study also showed that Themis2 interacts with Lyn, which was also upregulated in FcRL4$^+$ B cells. Altered expression of *THEMIS2* indicates that BCR signaling is modulated in FcRL4$^+$ B cells, although the implications for B cell activation of these cells remain to be elucidated.

Lymphomagenesis

FcRL4 is not only expressed by epithelium-associated B cells, but also by MALT lymphomas [9]. We therefore speculated that the highly proliferative [3], activated FcRL4$^+$ B cells may become neoplastic B cells. In support of this notion, we observed upregulation of several genes in FcRL4$^+$ B cells that are associated with lymphomagenesis. Firstly, expression levels of several NF-κB pathway genes were increased and all MALT lymphoma-associated gene translocations are associated with NF-κB activation [34]. An additional factor that may contribute to lymphomagenesis is upregulation of genes that promote cell survival (*TACI, BCL2A1*, *MLF2*) in FcRL4$^+$ B cells,

probably saving them from apoptosis. Furthermore, the most strongly downregulated gene in FcRL4+ B cells in the 'bulk' samples was *RGS16*. This gene was not differentially expressed in tonsillar or synovial FcRL4+ B cells [6,23]. RGS16 belongs to the family of regulators of G protein signaling (RGS), acting as GTPase activating proteins. RGS16 is involved in negative regulation of several oncogene pathways, including EGF/EGFR, MAPK, AKT/PI3K, RhoA, and SDF-1/CXCR4 in normal or cancer cell lines [35]. In addition, RGS16 was downregulated in mantle cell lymphoma B cells compared with naïve B cells [36], suggesting that the absence of RGS16 in FcRL4+ B cells may play a role in lymphomagenesis. The physiological role of RGS16 is, however, poorly understood and needs further investigation. A different gene that was strongly upregulated in FcRL4+ B cells is *IL27RA*. The expression level of this gene was previously shown to be increased in MALT lymphoma compared with other B cell lymphomas [37]. In mice, it was shown that IL-27 signals directly to B cells and promotes differentiation towards germinal center (GC) B cells via STAT1 [38]. In line with these findings, IL-27R was induced on GC B cells following CD40 stimulation and induced STAT1 phosphorylation in humans [39]. IL-27 could also induce T-bet expression in naïve and memory B cells [39,40]. Of interest, in the single cell samples, we identified a small amount of FcRL4+ B cells that expressed *AICDA*. Consistent with these findings, a previous study showed that FcRL4+ memory B cells have higher expression levels of *AICDA* than FcRL4- memory B cells [23]. The *AICDA* gene is coding for activation-induced cytidine (AID), which is essential for somatic hypermutation and isotype switching in GC B cells. Recently it was shown that AID can also target many other genes outside immunoglobulin loci, which may result in off-target, potentially oncogenic, mutations [41]. Prolonged expression of *AICDA* in FcRL4+ B cells may therefore contribute to transformation of FcRL4+ B cells towards neoplastic MALT lymphoma cells. The clear co-localization of neoplastic B cells with epithelial cells in MALT lymphomas suggests that this disease depends on the interaction between B cells and epithelial cells.

A limitation of our study is the small amount of single cells that were sorted and sequenced. Numbers of FcRL4+ cells within the infiltrate are relatively low, and for ethical reasons only a small-sized biopsy could be obtained for research purposes. Due to the stochasticity of gene expression, in combination with a high proliferation rate of FcRL4+ B cells [3], our differential expression analysis in single cells may be underpowered. Nonetheless, to our best knowledge this is the first study to reveal the gene expression profile of FcRL4+ B cells isolated from salivary gland tissue of pSS patients. We show that these cells exhibit all characteristics of chronically activated CD11c+ memory B cells, including integrin expression, which may be responsible for cross-talk with epithelial cells that form LELs. By interacting with epithelial cells, FcRL4+ B cells may contribute to pSS histopathology and hyposalivation. Lastly, we show that FcRL4+ B cells isolated from glandular tissue of pSS patients express anti-apoptotic factors that, combined with

ongoing somatic hypermutation and a high proliferative capacity, may put them at risk of lymphomagenesis.

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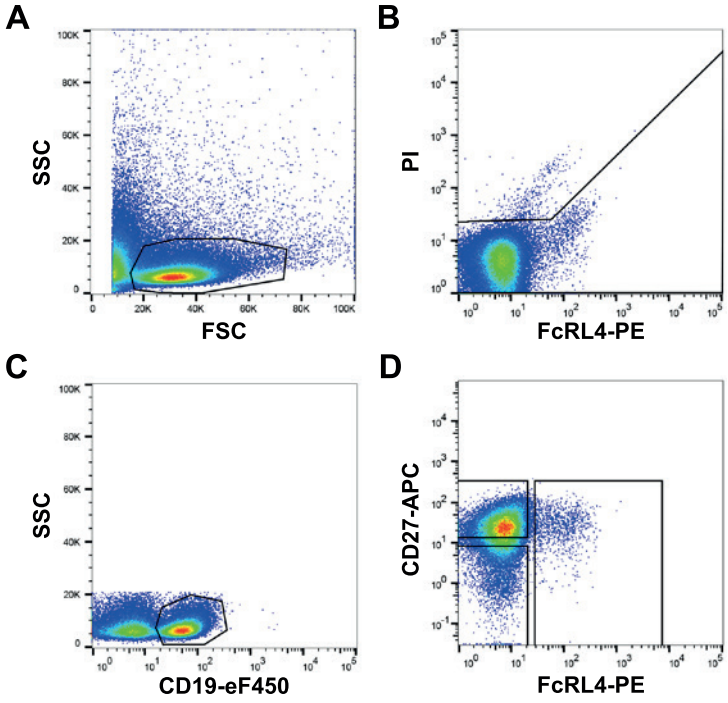
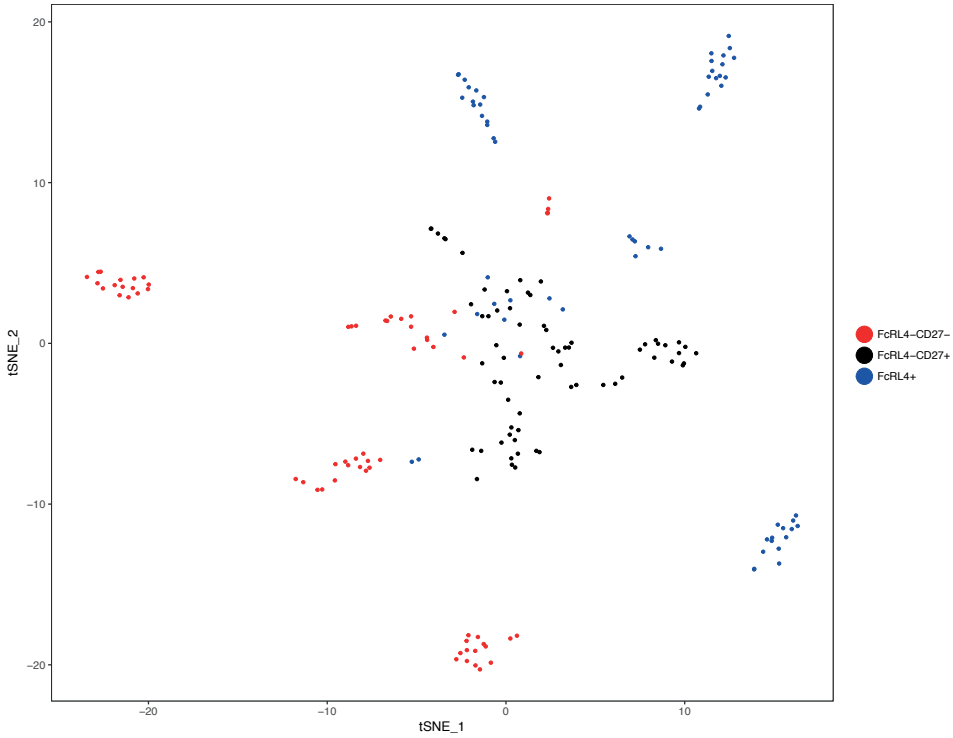


SUPPLEMENTARY FIGURE 1 | Gating strategy for sorting of B cell subsets from parotid gland tissue. (A) Lymphocytes were selected based on forward and side scatter properties. (B) Dead cells were excluded based on propidium iodide (PI) staining. (C) B cells were selected based on positive staining for CD19. (D) Three B cell subsets were sorted: $CD27^{-}FcRL4^{-}$, $CD27^{+}FcRL4^{-}$, and $CD27^{+/-}FcRL4^{+}$ cells.



SUPPLEMENTARY FIGURE 2 | T-distributed stochastic neighbor embedding (tSNE) plot of all single cell samples after unbiased clustering. Cells are colored by flow-cytometry based cell subsets.

5

SERUM IMMUNOGLOBULIN FREE LIGHT CHAINS ARE SENSITIVE BIOMARKERS FOR MONITORING DISEASE ACTIVITY AND TREATMENT RESPONSE IN PRIMARY SJÖGREN'S SYNDROME

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ABSTRACT

Objective

Serum immunoglobulin free light chains (FLC) are frequently elevated in B cell-mediated autoimmune diseases, including primary Sjögren's syndrome (pSS). The objective of this study was to assess if serum FLC can contribute to classification, MALT-lymphoma detection, monitoring of disease activity and treatment response in pSS.

Methods

Serum samples of 100 consecutive patients suspected of pSS were included. Forty-five patients fulfilled ACR-EULAR criteria for pSS. Additionally, samples of 17 pSS patients with MALT-lymphoma and longitudinal samples of pSS patients treated with rituximab (n=20), placebo (n=10) or abatacept (n=15) were included. Serum FLCκ/FLCλ was measured by nephelometry.

Results

At diagnosis, FLCκ and FLCλ serum levels were significantly higher in pSS compared to non-SS sicca patients. The FLCκ/FLCλ ratio was abnormal in 11% of pSS patients. In established MALT-pSS patients, without recent rituximab treatment (n=12), 50% had abnormal FLCκ/FLCλ ratios. FLC measurement had no additional value for pSS classification, compared to IgG and anti-SSA. FLC levels correlated significantly with systemic disease activity, assessed by EULAR Sjögren's syndrome disease activity index (ESSDAI) and clinical ESSDAI, both cross-sectionally and longitudinally following treatment. Treatment with rituximab or abatacept significantly lowered FLC levels. FLC show a large sensitivity to change and relative changes induced by treatment were higher compared with IgG.

Conclusion

Serum FLC is elevated in pSS, and abnormal FLCκ/FLCλ ratios may be indicative for the presence of MALT-lymhoma. FLC levels can be used as biomarker for systemic disease activity and monitoring treatment responses. FLC is sensitive to change and has more favorable kinetics than IgG.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disease which characteristically affects the salivary and lacrimal glands. B-cell hyperactivity is a major contributor to the pathology of pSS [1]. The involvement of B-cells in pSS pathogenesis is among others reflected by the presence of hypergammaglobulinemia and autoantibodies, such as anti-SSA/Ro, anti-SSB/La and rheumatoid factor (RF). The elevated intracellular levels of Bruton's tyrosine kinase in naive and memory B-cells further reflect their intrinsic, more activated state [2]. Also, pSS patients have an enhanced risk of developing mucosa-associated lymphoid tissue (MALT)-lymphoma, a subclass of malignant B-cell lymphoma [1,3]. In line with this role for B-cells in pSS pathogenesis, B-cell depletion therapy with rituximab shows favorable effects on salivary gland architecture and extraglandular manifestations of pSS [4]. However, not all patients benefit from this treatment, as shown by two large randomized controlled trials [5,6]. Validated biological markers for monitoring clinical response to therapy and/or disease activity in pSS are lacking. Promising markers are serological parameters of B-cell activity, including BAFF (B-cell Activation of the TNF Family, also named BLyS), CXCL13, β2-microglobulin and immunoglobulin free light chains (FLC). These markers are fairly associated with systemic disease activity in pSS patients [7,8].

FLC comprise kappa (κ) and lambda (λ) light chains, which are produced in excess compared to heavy chains during immunoglobulin synthesis by B-cells, plasmablasts and plasma cells. The surplus of light chains is secreted as FLC into serum [9]. The half-life of FLC in serum is very short (2-6 hours) compared to complete immunoglobulins and therefore the presence of FLC may reflect actual B-cell activity. FLC may have specific biological functions, which include binding to antigens on antigen-presenting cells and inhibition of apoptosis of neutrophils [10–12]. FLC serum levels correlate positively with other serological markers of B-cell activity and most strongly with serum IgG [13]. Levels of polyclonal FLC are not only elevated in pSS compared to healthy controls, but also in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [13–15]. In patients with SLE, FLC levels correlate with disease activity and are elevated in urine prior to relapse of symptoms [14,16]. However, the clinical relevance of serum FLC levels as a biomarker in pSS is unclear. The objectives of this study were to assess if serum FLC can contribute to classification, MALT-lymphoma detection, monitoring of disease activity and treatment response in pSS.

PATIENTS AND METHODS

Study population

Consecutive patients (n=129), referred to the Sjögren Expertise Center of the University Medical Center Groningen (UMCG) for suspicion of pSS, were screened for eligibility. Exclusion criteria were age<18 years, an incomplete diagnostic work-up, diagnosis of another systemic auto-immune disease, hepatitis C positivity and renal impairment (eGFR<60 mL/min, MDRD formula), which influences serum FLC levels [9]. Patients that fulfilled 2016 ACR-EULAR criteria for pSS were classified as pSS patients [17], and the remaining patients as non-SS sicca patients. Serum samples of 18 consecutive pSS patients with a current or pre-existing diagnosis of salivary gland MALT-lymphoma, who were enrolled as a subgroup in our Registry of Sjögren syndrome in UMCG-Longitudinal (RESULT) cohort, were also screened for eligibility. Of these 18 patients, one was excluded from FLC analysis because of renal impairment. The remaining 17 pSS-MALT patients were divided in a RTX-treatment group (rituximab treatment <1 year before inclusion) and a no-treatment group (untreated patients and patients who were treated with rituximab >1 year before inclusion). Informed consent was obtained from all patients according to the Declaration of Helsinki and the studies were approved by the Medical Research Ethics Committee of the UMCG (METc2013.066/METc2014.491).

Additionally, longitudinal serum samples of pSS patients that participated in our previously reported double-blind, randomized controlled trial with rituximab (1000 mg, days 1 and 15, n=30 with 2:1 randomization for rituximab and placebo) and open-label study with abatacept (~10 mg/kg of body weight, days 1, 15 and 29 and every 4 weeks thereafter, n=15) were included [18,19]. One patient in the placebo-arm of the rituximab study was lost to follow-up after week 12 and therefore no samples were available from this patient at week 24, 36 and 48. To minimize the risk of infusion reactions and serum sickness, all patients in the rituximab- and placebo-arm were treated with methylprednisolone (100 mg IV), acetaminophen (1000 mg orally) and clemastine (2 mg IV) prior to each infusion, and received oral prednisone (60 mg on days 1&2, 30 mg on days 3&4 and 15 mg on day 15). Except for these precautionary medications, treatment with concomitant immunosuppressants, including hydroxychloroquine and glucocorticoids, was discontinued during the study. All patients that participated in the rituximab and abatacept studies also fulfilled, retrospectively, the ACR-EULAR criteria. The EULAR Sjögren's syndrome disease activity index (ESSDAI) and clinESSDAI (ESSDAI without the biological domain) were completed in all pSS patients [20,21].

FLC measurement

Stored serum samples (-80°C) were thawed and FLC (κ and λ) were measured by quantitative turbidimetry using the Freelite assay and the Optilite analyzer (Binding

Site, UK). The Freelite assay estimates FLCκ and FLCλ by separate immunoassays based on affinity-purified polyclonal antibodies coated onto latex particles. Samples from the MALT-lymphoma group and the rituximab study were measured by quantitative nephelometry using the Freelite assay and the BNProSpec analyzer (Siemens AG, Germany), as part of the diagnostic work-up. Reference intervals were 3.3–19.4 mg/L for FLCκ, 5.7-26.3 mg/L for FLCλ, and 0.26-1.65 for the κ/λ ratio, according to the 95% percentile range provided by the manufacturer. An abnormal FLC level was defined as an abnormal kappa and/or lambda level and/or abnormal κ/λ ratio.

Statistical analysis

Continuous data are presented as medians and interquartile ranges. Mann-Whitney U test was used to compare FLC levels between different groups. Predictor analyses of pSS classification were performed using binary logistic regression. Multivariate analysis was performed using the forward Wald method for inclusion of predictors that had a P-value <0.05 in the univariate analysis. The explained variance is presented as Nagelkerke's R square (R2). Residual statistics were assessed and outlier cases were identified based on Cook's distance >1, or standard and normalized residual values outside the cut-off values of ±1.96 and ±2.58, respectively.

Cross-sectional correlations between serum FLC and clinical and biological parameters (i.e., ESSDAI, clinESSDAI, IgG) were evaluated with Spearman's correlation coefficient. Longitudinal correlations between FLC and clinical parameters were analyzed with generalized estimating equations (GEE). GEE were used to analyze changes in FLC levels over time, namely during B-cell depletion (week 0-24), B-cell repopulation (week 24-48), during abatacept treatment (week 0-24) and after cessation of abatacept treatment (week 24-38). For the placebo group, identical time points were analyzed as for the rituximab group. To evaluate sensitivity to change, the standardized response mean (SRM) was calculated as the mean change score divided by the SD of the change score, between baseline and each consecutive time point. SRM <0.5 were interpreted as small, 0.5–0.8 as moderate, and >0.8 as large [22]. P-values <0.05 were considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics 23 (SPSS, USA).

RESULTS

From the diagnostic cohort, 100 out of 129 patients were included in the analyses. Reasons for exclusion were an incomplete diagnostic work-up (n=8), presence of another systemic auto-immune disease (n=12), missing serum samples (n=5), hepatitis C infection (n=2), or renal impairment (n=2). There were no patients with renal impairment in the treatment groups (rituximab, abatacept) at baseline or during

follow-up. Baseline patient's characteristics of the different cohorts are summarized in table 1. Detailed clinical characteristics of the MALT-lymphoma group are described in supplementary table 1.

Elevated FLC levels and κ/λ ratio in pSS patients at the time of diagnosis

Of the included patients from the diagnostic cohort, 45 (45%) patients were classified as having pSS. Twenty-nine (64%) of these pSS patients had abnormal FLC levels. Both FLCκ and FLCλ levels were significantly increased in pSS compared with non-SS sicca patients (figures 1A and 1B, P<0.001). In pSS, FLCκ and FLCλ levels were above the upper reference limits in 58% and 44% of patients, respectively. FLC levels were higher in anti-SSA positive, compared with anti-SSA negative patients (P=0.037 for FLCκ, P=0.064 for FLCλ). In addition to absolute levels of FLC, the κ/λ ratio was assessed. A small but significant increase in the κ/λ ratio was observed in pSS, compared with non-SS sicca patients (figure 1C, P=0.009), indicating a slightly higher production of FLCκ versus FLCλ. There was no significant difference in κ/λ ratio between pSS patients with or without anti-SSA antibodies.

In five (11%) pSS patients and three (5%) non-SS sicca patients, a κ/λ ratio above the upper cut-off value was found. One of these pSS patients was diagnosed with MALT-lymphoma. This patient also presented with abnormal levels of RF (>200 IU/mL), IgG (25 mg/L), and weak type III cryoglobulinemia (polyclonal), but without lymphopenia or low C4. A second pSS patient with an abnormal κ/λ ratio had an IgG level of 32 mg/L and developed urticarial vasculitis and unilateral parotid gland swelling a few months after inclusion. A third pSS patient with an abnormal κ/λ ratio developed progressive neuropathy with lymphopenia, low C4 level and weak type I cryoglobulinemia (monoclonal) one year later during follow-up. In the other two pSS patients and in the three non-SS sicca patients with an abnormal κ/λ ratio, risk factors for lymphoma such as cryoglobulinemia, lymphopenia or low complement levels were not detected. Together, these results indicate that an altered κ/λ ratio may indicate or precede severe clinical manifestations of pSS.

TABLE 1 | Baseline characteristics of different study cohorts.

Characteristic	Diagnostic cohort (n=100)		MALT- lymphoma cohort (n=17)	Rituximab trial (n=30)		Abatacept trial (n=15)
	pSS (n=45)	non-SS sicca (n=55)		RTX (n=20)	Placebo (n=10)	
Age, median (IQR), years	54 (46-63)	50 (40-56)	60 (50-64)	42 (32-53)	37 (32-60)	43 (32-51)
Female gender, n (%)	43 (96)	47 (86)	16 (94)	19 (95)	10 (100)	12 (80)
ESSDAI, median (IQR)	4 (1-9)	-	4 (1-15)	8 (6-11)	7 (5-9)	11 (8-14)
clinESSDAI, median (IQR)	4 (0-10)	-	4 (0-13)	7 (5-11)	5 (5-8)	11 (9-17)
IgG (g/L), median (IQR)	16 (12-20)	10 (9-12)	13 (9-17)	22 (19-26)	23 (16-27)	20 (15-27)
Anti-Ro/SSA positive, n (%)	34 (76)	3 (6)	16 (94)	20 (100)	10 (100)	15 (100)
Anti-La/SSB positive, n (%)	19 (42)	0 (0)	6 (35)	14 (70)	8 (80)	12 (80)
Biologic activity, n (%)	25 (56)	10 (18)	11 (65)	17 (85)	9 (90)	11 (73)
Treatment with corticosteroids, n (%)	2 (4)	2 (4)	2 (12)	0 (0)*	0 (0)*	0 (0)*
Treatment with DMARD, n (%)	7 (16)	3 (5)	7 (41)	0 (0)*	0 (0)*	0 (0)*
Kappa FLC mg/L, median (IQR)	22 (16-36)	15 (11-18)	24 (16-37)	23 (17-45)**	21 (15-27)**	38 (28-44)**
Lambda FLC mg/L, median (IQR)	21 (15-31)	15 (13-18)	15 (10-22)	26 (19-46)**	23 (19-24)**	30 (23-41)**
Ratio kappa/lambda	1.15 (0.89-1.51)	0.97 (0.78-1.20)	1.44 (1.15-1.95)	0.96 (0.75-1.24)**	0.98 (0.74-1.11)**	1.11 (0.86-1.52)**

*Treatment with prednisolone, hydroxychloroquine or other traditional disease-modifying anti-rheumatic drugs (DMARD) had to be discontinued at least 1 month before baseline. **Values at baseline, i.e. before start of treatment, are displayed. MALT: Mucosa-associated lymphoid tissue; IQR: Interquartile range; ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index; clinESSDAI: ESSDAI without the biological domain.

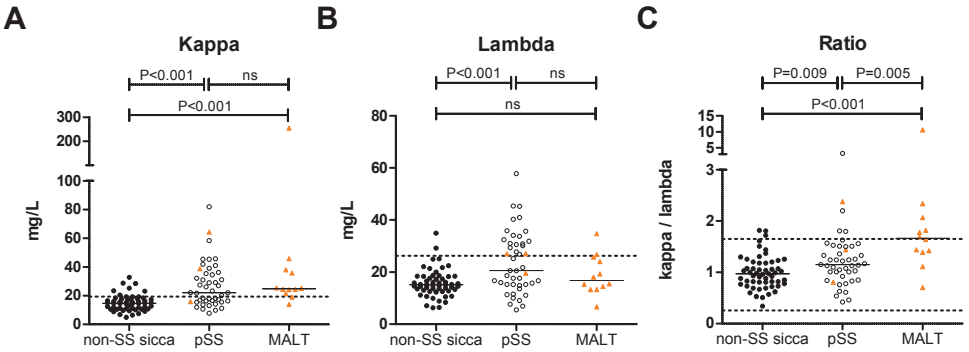

FIGURE 1 | Serum levels of FLCκ and FLCλ and the κ/λ ratio in pSS patients and sicca patients. FLC levels in a diagnostic cohort consisting of non-SS sicca patients (n=55) and pSS patients (n=45), classified according to the ACR-EULAR criteria, are displayed. In addition, results from established pSS patients with mucosa-associated lymphoid tissue (MALT)-lymphoma located in the salivary glands, without or with current/recent (<1 year) B-cell depletion therapy, are displayed (n=12 and n=5, respectively). Horizontal lines indicate the median. Dashed horizontal lines indicate the cut-off value(s). Orange triangles represent patients with salivary gland MALT-lymphoma without current/recent B cell depletion therapy, blue triangles represent patients with salivary gland MALT-lymphoma who have been treated with B cell depletion therapy <1 year before inclusion. Mann-Whitney U test was used for statistical comparisons between pSS and non-SS sicca patients. The one-way ANOVA (Kruskal Wallis) test was used to compare pSS, untreated pSS-MALT and treated pSS-MALT patients. P-values <0.05 were considered significant. Ns: Not significant.

Abnormal κ/λ ratios in MALT-lymphoma patients

Because an abnormal κ/λ ratio was observed in a MALT-lymphoma patient in our diagnostic cohort, these ratios were also evaluated in established pSS patients with salivary gland MALT-lymphoma. Seventeen MALT-lymphoma patients were included, and five patients were analyzed separately because of recent or current B cell depletion therapy. Of the 12 patients who were not treated with rituximab, six (50%) patients had an abnormal κ/λ ratio (figure 1C). In only one of these six patients a weak monoclonal immunoglobulin (M-protein) was detected by immunofixation. Of the five patients who were recently treated with rituximab, only one had an abnormal κ/λ ratio (data not shown). The latter patient with a κ/λ ratio of 6.2 received maintenance treatment with rituximab every half year, because of recurrent symptoms of vasculitis and polyneuropathy in the last three years.

FLC versus IgG measurement for prediction of pSS

We next investigated if FLCκ and FLCλ could predict pSS classification by logistic regression analysis. Univariate logistic regression analysis showed that FLCκ and FLCλ are both significant predictors of fulfilling the ACR-EULAR criteria for pSS (R^2: 0.374 and 0.254, respectively). However, the explained variances for IgG and anti-SSA positivity were higher (R^2: 0.521 and 0.605, respectively). After inclusion of the variables FLCκ, FLCλ,

IgG and anti-SSA positivity in multivariate analysis, IgG and anti-SSA positivity were the only independent predictors of pSS classification (R^2: 0.696). When only FLCκ, FLCλ, and anti-SSA were included in the multivariate analysis, FLCκ contributed significantly to the model (supplementary table 2). Exclusion of 5 outliers (see Methods) improved the predictive value to 0.863, but did not change the independent variables included in the models (data not shown). Together, our results indicate that FLC levels are neither superior nor additional to IgG and anti-SSA positivity as a classification biomarker for pSS.

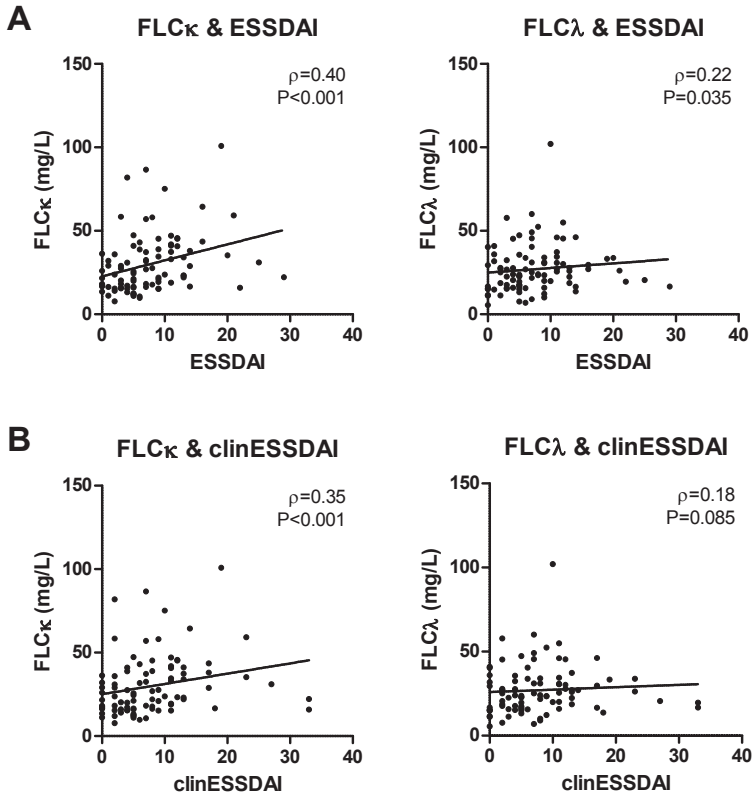

FIGURE 2 | Correlations between FLC and systemic disease activity. Correlations between FLC and systemic disease activity, as measured by ESSDAI and clinESSDAI, in 90 pSS patients classified according to ACR-EULAR criteria. Patients with pSS from the diagnostic cohort (n=45) and baseline data from the rituximab (n=30) and abatacept (n=15) cohorts were combined. Correlations were evaluated with Spearman's correlation coefficient (ρ).

Association between FLC levels and systemic disease activity at baseline

Previous studies have suggested that FLC levels correlate positively with extraglandular involvement in pSS patients [7,13]. Consistent with this finding, combined data from our diagnostic cohort and baseline data from the rituximab and abatacept trials, in which

ESSDAI scores were relatively high (table 1), indicated that FLCκ, and to a lesser extent also FLCλ, correlated significantly with ESSDAI scores (figure 2A). The ESSDAI domain that showed the highest correlation with FLC levels was the biological domain, which can be explained by the strong correlations between IgG and FLCκ and FLCλ (ρ=0.685 and ρ=0.621, respectively). Therefore, also correlations between FLC and clinESSDAI were assessed and only FLCκ was significantly correlated to clinESSDAI scores, although a trend was seen for FLCλ (figure 2B).

FLC levels are affected by immunomodulatory treatment

Rituximab

In the group of rituximab-treated and placebo-treated patients, 19 (63%) patients had abnormal FLC levels at baseline, which is comparable to the diagnostic cohort. None of the patients had an abnormal κ/λ ratio. Median values are displayed in table 1. No patients had monoclonal gammapathy, and no MALT-lymphoma patients were included in this treatment study. Rituximab reduced both FLCκ and FLCλ levels significantly over time (figure 3A). In placebo-treated patients, FLC also decreased significantly over time (figure 3B), which could be attributed to a drop only at week 5. In line with this notion, the decrease in FLC in the rituximab group over time was stronger, of longer duration, and significantly different from the placebo group ($P<0.001$ for both FLCκ and FLCλ). Furthermore, during B cell repopulation in the rituximab group, FLCκ and FLCλ levels increased to baseline values (figure 3A), whereas no changes were seen in the placebo group between weeks 24 and 48 (figure 3B). Relative changes in FLCκ and FLCλ after 24 weeks of treatment with rituximab were higher compared to IgG (median Δ -28%, -24% and -21%, respectively). SRM values for responsiveness were large (>0.8) for both FLC in the rituximab group, persisting until week 36 for FLCκ and week 24 for FLCλ (figure 4A). In the placebo group, a high SRM was found only at week 5 (figure 4B).

Abatacept

At baseline, 14 (93%) patients had abnormal FLC levels, which is higher compared to the diagnostic cohort and the rituximab group. Three (15%) patients had an abnormal κ/λ ratio. Serum FLC levels were reduced by abatacept treatment (figure 3C). Levels of FLCκ decreased to a larger extent than FLCλ (figure 3C). Median relative changes between baseline and week 24 for FLCκ, FLCλ and IgG were -18%, -10% and -12%, respectively, indicating that FLCκ was most strongly reduced by abatacept treatment. SRM values were larger for FLCκ than FLCλ (figure 4C).

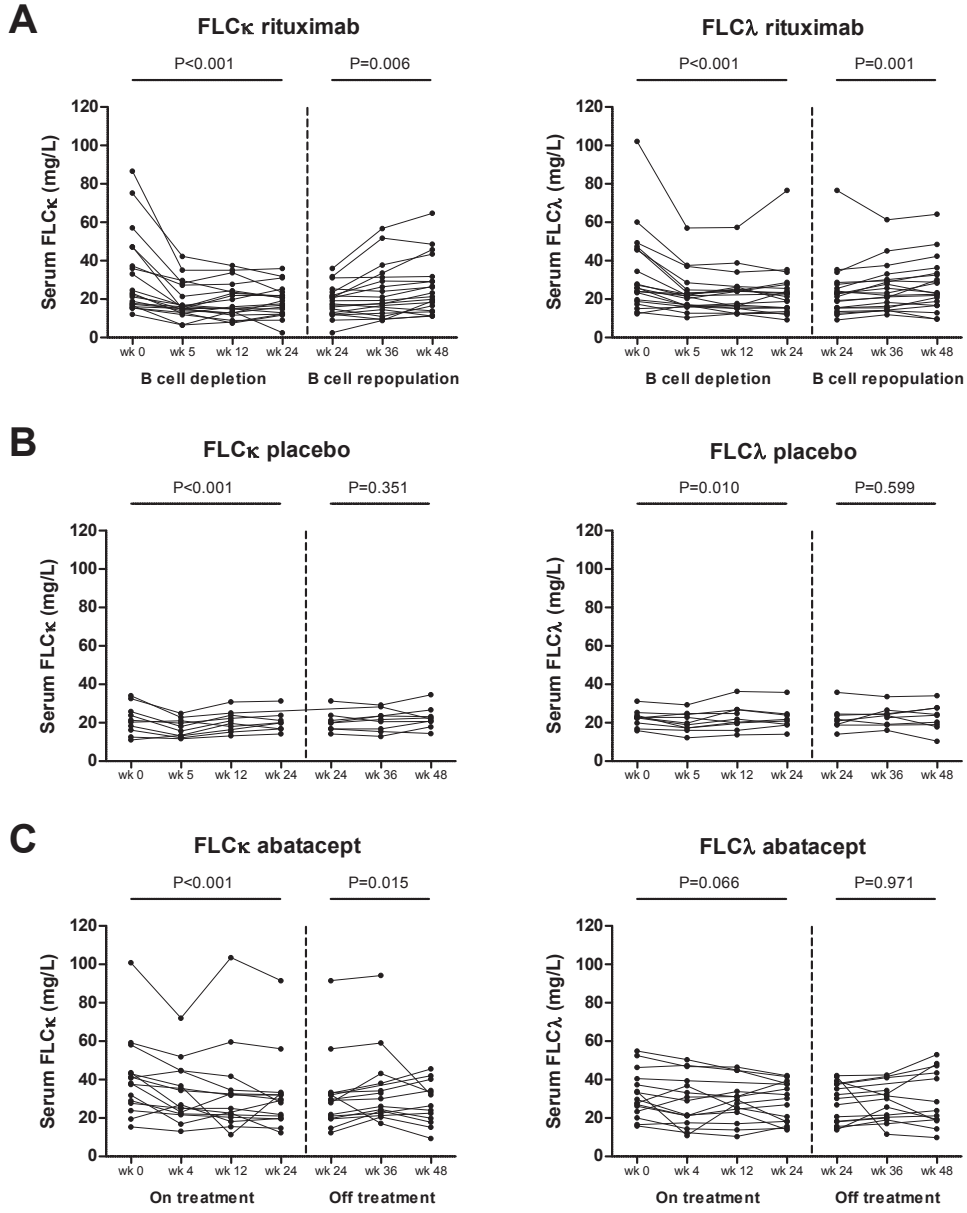

FIGURE 3 | FLC levels are lowered by immunomodulatory treatment. (A) FLCκ and FLCλ serum levels during B cell depletion (week 0-24) and B cell repopulation (week 24-48). (B) FLCκ and FLCλ serum levels in placebo-treated patients at the same time points as rituximab-treated patients. (C) FLCκ and FLCλ serum levels during abatacept treatment (week 0-24) and after cessation of treatment (week-24-48). P-values were calculated using generalized estimating equations with log-transformation of dependent variables.

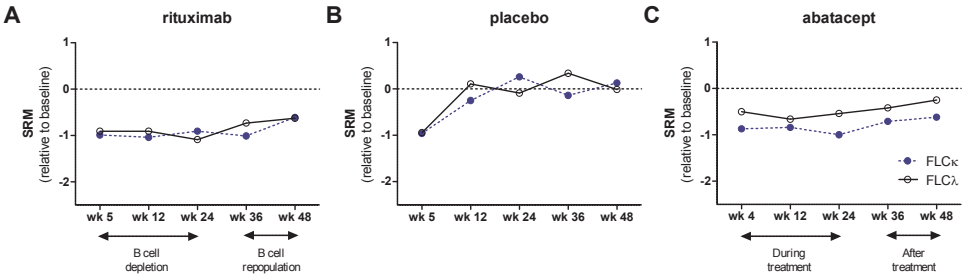

FIGURE 4 | Responsiveness of FLCκ and FLCλ in pSS patients treated with rituximab, placebo or abatacept. Standardized response means (SRM) are displayed for each treatment group at different time points during treatment (week 4/5, week 12, week 24) or after treatment (week 36 and 48). A negative SRM indicates lowering of FLC. SRM <0.5 is interpreted as small, 0.5–0.8 as moderate, and >0.8 as large.

Associations between changes in FLC levels and systemic disease activity during treatment

Finally, we assessed if the observed changes in FLC levels correlated with clinical and biological parameters over time during treatment, namely the period of B cell depletion by rituximab, and the period of treatment with abatacept (both week 0-24). We performed the same analyses for the period after treatment (week 24-48). The decrease in FLCκ and FLCλ in the rituximab group was significantly associated with lowering of ESSDAI and clinESSDAI scores from baseline to week 24 (table 2). Weaker correlations were observed in the placebo group (table 2). In the abatacept group, only FLCκ was significantly associated with ESSDAI and clinESSDAI scores over time during treatment (week 0-24).

TABLE 2 | Associations between FLC and systemic disease activity scores during treatment.

	ESSDAI		**ClinESSDAI**		**ESSDAI**		**ClinESSDAI**	
	During treatment (week 0-24)				**After treatment (week 24-48)**			
	Beta	***P-value***	***Beta***	***P-value***	***Beta***	***P-value***	***Beta***	***P-value***
FLCκ rituximab	0.030	**<0.001**	0.024	**<0.001**	0.015	**<0.001**	0.011	**<0.001**
FLCκ placebo	0.015	**<0.001**	0.009	0.079	0.009	0.138	0.004	0.082
FLCκ abatacept	0.008	**0.003**	0.007	**0.002**	-0.004	0.425	-0.003	0.324
FLCλ rituximab	0.018	**<0.001**	0.015	**<0.001**	0.007	**0.020**	0.005	**0.037**
FLCλ placebo	0.005	0.052	0.005	**0.011**	0.013	**0.007**	0.007	**0.012**
FLCλ abatacept	0.002	0.188	0.002	0.235	-0.006	0.236	-0.006	0.141

Longitudinal analysis were conducted for the period during treatment (week 0-24) and after treatment (week 24-48). Logarithmic transformation of FLC values was performed, and transformed values were entered into the Generalized Estimating Equation (GEE) models. Beta represents the regression coefficient (log-transformed). ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome Disease Activity Index; clinESSDAI: ESSDAI without inclusion of the biological domain. Significant P-values are displayed in bold.

Also after treatment, in the rituximab group, FLCκ and FLCλ levels correlated significantly with ESSDAI/clinESSDAI scores (table 2), suggesting that the increase in both parameters after treatment occurs in a similar manner.

DISCUSSION

Serum FLCs are biomarkers for actual B-cell activity and are frequently elevated in systemic autoimmune diseases [13,15]. This study shows that serum FLCκ and FLCλ levels are frequently elevated in pSS patients, compared to non-SS sicca patients, at the time of diagnosis. Importantly, we found that an abnormal FLC κ/λ ratio can be indicative for the presence of MALT-lymphoma. This study further shows that FLC levels rapidly decrease following treatment with rituximab or abatacept, and that these levels are associated with systemic disease activity at baseline and longitudinally in response to treatment.

In our diagnostic cohort, FLCκ and FLCλ levels were abnormal in 58% and 44% of patients, respectively. This percentage is considerably higher than the previously reported percentages of 22-24% by Gottenberg et al. [7,13]. This difference may be explained by higher B-cell activity in our cohort, reflected by higher IgG levels, and/or by the lower frequency of immunosuppressive drug use in our cohort. In line with a previous study by Sudzius et al. [23], we observed that increased FLC levels were more pronounced in anti-SSA-positive, compared with anti-SSA-negative pSS patients, suggesting that patients with anti-SSA antibodies exhibit higher B-cell activity and hence produce more FLC. We also found a subtle, but significant increase in the κ/λ ratio in pSS patients, though only 11% of the pSS patients had an abnormal ratio according to the 95-percentile range [7]. The reason for this skewing is not clear, but one possibility is that it is due to the autoantibody status, indicating that autoreactive B-cells might express relatively more kappa than lambda chains. However, no difference in the κ/λ ratio was observed between anti-SSA-positive and -negative patients, in line with a previous report [23]. In addition to anti-SSA/-SSB antibodies, pSS patients frequently produce RF. RF-positivity in pSS is associated with a higher risk of lymphoma development [24]. Bende et al. showed that 41% of salivary gland MALT-lymphomas expressed B-cell receptors with strong RF-homology [25]. Interestingly, the vast majority of these lymphomas was typed as κ-predominant by immunohistochemistry. These data suggest that RF-producing B cells may contribute to the skewed κ/λ ratio in a subgroup of pSS patients.

Because one of the pSS patients with an abnormal κ/λ ratio in our diagnostic cohort was diagnosed with MALT-lymphoma, we evaluated FLC levels in a cohort of established pSS patients with MALT-lymphoma located in the salivary gland. A previous study by Witzig et al. revealed elevated FLC levels in 31% of MALT-lymphoma patients,

but lymphoma localization was not specified [26]. Whether also salivary gland MALT-lymphomas in pSS patients present with abnormal FLC levels and/or ratios, is to the best of our knowledge not known. Our study shows that the κ/λ ratio was increased in 50% of patients with salivary gland MALT-lymphoma. In only one of the 12 (8%) pSS patients with MALT lymphoma a weak M-protein was detectable, indicating that secretion of complete immunoglobulins occurs only infrequently. A similar low frequency (9%) of M-protein in pSS-MALT patients was revealed by Wöhrer et al. [27], whereas we showed previously that M-proteins were present in 8 out 35 (23%) pSS-MALT patients [28]. Recent studies showed that the frequencies of monoclonal gammopathy in larger cohort of pSS patients were 7.4%-22% [29,30]. However, only few of the patients with M-proteins (0-6%) developed salivary gland MALT-lymphoma (follow-up time 6.3-10 years). These data strongly argue that the κ/λ ratio in serum may be more useful as indicator for the presence of MALT-lymphoma than finding a M-protein. This situation is analogous to patients with a monoclonal gammopathy of unknown significance (MGUS) in which the risk of progression to a malignant monoclonal gammopathy is higher in patients with an abnormal κ/λ ratio than in patients with a normal ratio [31]. In contrast to other pSS-lymphoma cohorts [8,32], the median ESSDAI score in our pSS-MALT cohort was relatively low. This may be explained by the fact that in our clinical work-up parotid gland biopsies are taken for diagnostic purposes. This setting results in unexpected detection of MALT-lymphoma in patients with low disease activity, as shown previously [28]. A second, not-mutually exclusive, explanation could be that both patients with a current or pre-existing MALT-lymphoma diagnosis were included. The finding that patients with a pre-existing MALT-lymphoma diagnosis frequently present with abnormal κ/λ ratios suggests that this biomarker may also be useful as indicator of MALT-lymphoma persistence and/or recurrence. Recurrence appears in 29-35% of the parotid gland MALT-lymphoma cases [28,33].

In addition to the findings in pSS-MALT patients, our study confirmed that abnormal FLC levels in pSS are associated with higher systemic disease activity, as measured by ESSDAI scores [7]. The biological domain of the ESSDAI score also includes elevated serum IgG levels, which obviously may bias the correlation between FLC levels and ESSDAI. For this reason we correlated FLC levels not only to ESSDAI, but also to clinESSDAI scores [20]. Interestingly, FLCκ was significantly and more strongly correlated to both ESSDAI and clinESSDAI scores than FLCλ. Thus, increased disease activity of pSS is not only associated with increased polyclonal B-cell activity, but remarkably also with preferential expansion of the kappa subtype, which is confirmed in other studies in pSS [7,8,13]. The reason for this preferential expansion remains obscure.

Besides a role as biomarker for systemic disease activity, FLC are potentially useful in monitoring the effect of immunomodulatory treatment on B-cell activity in pSS patients. Therefore, the effect of treatment with rituximab and abatacept on serum FLC

levels was evaluated. Rituximab treatment significantly reduced FLCκ and FLCλ levels, reflecting the decreased number of antibody-secreting cells (i.e., B-cells, plasmablasts, plasma cells). A reduction in FLC was also seen in the placebo group at week 5, likely due to corticosteroid treatment around the rituximab infusions, but a significantly stronger decrease was observed in the rituximab group. The decrease in FLC correlated significantly with lowering of ESSDAI and clinESSDAI during B-cell depletion. These findings are consistent with studies in RA and SLE, showing a correlation between normalization of FLC levels and disease activity upon rituximab treatment [34,35]. We found that abatacept treatment also significantly reduced FLCκ and, to a smaller extent, FLCλ levels. FLCκ levels were significantly associated with ESSDAI and clinESSDAI scores over time during treatment. As B-cells are not directly targeted by abatacept, the observed decrease in FLC is likely a result of reduced T-cell help to B-cells. This is further substantiated by the previously observed decrease in serum levels of IgG, anti-SSA antibodies and RF, as well as circulating plasmablasts [19,36]. Additionally, our study reveals that FLC have a large sensitivity to change, assessed by SRM, and that relative changes in FLCκ were higher compared with IgG. An advantage of monitoring FLC is that changes in serum levels are more rapidly induced after initiation of treatment, compared with IgG. FLC have a half-life of several hours, while IgG has a half-life of several weeks [9]. Therefore, B-cell activity can be monitored by FLC levels without the delay that is seen for IgG.

In summary, this study shows for the first time that the κ/λ ratio in serum is abnormal in 50% of salivary gland MALT-lymphoma patients. Abnormal ratios should be taken seriously and might be indicative for the presence, persistence and/or recurrence of MALT lymphoma. Prospective analysis of the κ/λ ratio in pSS patients is necessary to confirm its potential role as predictor of MALT-lymphoma development, and to compare sensitivity with M-protein analysis. Moreover, our data underline the pathogenic role of B-cell hyperactivity, and particularly kappa-expressing B-cells, in pSS. Finally, we suggest that FLC are useful biomarkers to monitor the effect of immunomodulatory treatment on B-cell activity.

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SUPPLEMENTARY MATERIALS

SUPPLEMENTARY TABLE 1 | Characteristics of the pSS-MALT patients at inclusion.

	Age	Sex	FLCκ (mg/L)	FLCλ (mg/L)	FLC κ/λ ratio	Anti-SSA positive	IgG (g/L)	RF (IU/mL)	M-protein	Cryo-glubulins	Low complement (C3/C4)	Parotid gland swelling	ESSDAI*	ClinESSDAI*	Time since pSS diagnosis (years)	Time since MALT diagnosis (years)	Location MALT	Current RTX treatment*	Previous MALT treatment
1	50	M	24.5	14.5	**1.69**	Yes	14.2	6.2	Neg	Neg	Low C3	No	15	14	1	1	Parotid right	No	None
2	70	F	25.4	15.5	1.64	Yes	13.2	10	Neg	Neg	Low C3	Yes*	15	14	7	5	Parotid right + left	No	Radiotherapy
3	68	F	35.8	15.3	**2.34**	Yes	18.5	45	Oligo	type III (weak)	No	No	7	5	27	12	Parotid right	No	None
4	50	F	24.2	13.3	**1.82**	Yes	11.2	14	Neg	type III (weak)	No	No	2	0	8	3	Parotid left+stomach	No	RCP
5	60	F	45.8	25.8	**1.78**	Yes	19.8	100	Neg	Neg	No	No	1	0	12	11	Parotid left	No	RCP
6	29	F	19.1	13.3	1.44	Yes	10.1	54	Neg	Neg	Low C3	No	1	0	3	2	Parotid right	No	RCP
7	61	F	25.2	18.1	1.39	Yes	13.4	38	Neg	Neg	No	Yes	10	12	4	3	Parotid right	No	None
8	61	F	38.1	26.8	1.42	Yes	14.2	6.3	Neg	type II poly-Ig	No	No	2	2	25	11	Parotid right	No	RCP
9	60	F	254.0	24.0	**10.58**	Yes	30.7	>200	Neg	type II	No	Yes	30	29	0	0	Parotid right	No	None
10	49	F	21.5	19.3	1.11	Yes	17.9	0	Neg	Neg	No	No	1	0	4	3	Parotid right	No	None
11	65	F	13.9	6.7	**2.07**	Yes	11.5	39	Neg	Neg	No	No	0	0	6	6	Parotid left	No	None
12	61	F	24.3	34.8	0.7	Yes	16.3	>200	IgG-λ (weak)	type II	No	Yes	24	23	2	0	Parotid right	No	None
13	49	F	10.9	9.3	1.17	No	8.6	0	Neg	Neg	No	No	0	0	8	2	Parotid right	Yes	RCP, radiotherapy
14	75	F	7.7	7.2	1.07	Yes	6.2	2.7	Neg	Neg	No	No	4	4	7	3	Parotid right	Yes**	RCP
15	49	F	18.0	14.2	1.27	Yes	11	4.3	Neg	type I (weak)	No	No	14	12	4	2	Parotid left	Yes**	RCP
16	62	F	12.5	11.2	1.12	Yes	6.6	8.1	Neg	Neg	No	No	2	2	22	12	Parotid left	Yes**	RCP
17	54	F	39.6	6.4	**6.2**	Yes	6.7	17	Neg	type II	Low C4	No	14	12	3	3	Parotid left	Yes	RCP

Abnormal FLC ratios are displayed in bold. *Because of gland enlargement due to lymphoma, lymphoma was scored in the lymphadenopathy and lymphoma domain and parotid gland swelling was not scored in the glandular domain in this patient. **Rituximab was continued for treatment of extraglandular manifestations, after finishing induction treatment with RCP. The treated MALT-lymphoma in these patients was considered in remission and therefore lymphoma was not scored positively in the ESSDAI. FLC: Serum immunoglobulin free light chains; RF: rheumatoid factor (IgM); M-protein: monoclonal protein; ESSDAI: European League Against Rheumatism Sjögren's Syndrome Disease Activity Index. ClinESSDAI: Clinical ESSDAI (ESSDAI without inclusion of the biological domain); MALT: mucosa-associated lymphoid tissue (lymphoma); RTX: rituximab; RCP: rituximab, cyclophosphamide, prednisone.

SUPPLEMENTARY TABLE 2 | Biomarkers associated with fulfillment of ACR-EULAR classification for primary Sjögren's syndrome.

	Univariate analysis		Multivariate analysis		
Biomarker	**Nagelkerke R^2**	**P**	**Model 1 P**	**Model 2 P**	**Model 3 P**
FLCκ	0.374	<0.001	ns	0.035	ns
FLCλ	0.254	<0.001	ns	ns	ns
IgG	0.521	<0.001	<0.001	-	0.001
Anti-SSA*	0.605	<0.001	-	<0.001	<0.001

*Anti-SSA status was entered as a binary variable. All other independent variables were entered as continuous variables. Ns: Not significant.

PART II

T CELL-DEPENDENT B CELL HYPERACTIVITY: TARGET FOR TREATMENT?

6

B CELL DEPLETION THERAPY NORMALIZES CIRCULATING FOLLICULAR TH CELLS IN PRIMARY SJÖGREN'S SYNDROME

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ABSTRACT

Objective

To assess the effect of B cell depletion therapy on effector $CD4^+$ T cell homeostasis and its relation to objective measures of disease activity in patients with primary Sjögren's syndrome (pSS).

Methods

Twenty-four patients with pSS treated with rituximab (RTX) and 24 healthy controls (HC) were included. Frequencies of circulating effector $CD4^+$ T cell subsets were examined by flow cytometry at baseline and 16, 24, 36, and 48 weeks after the first RTX infusion. Th1, Th2, follicular T helper (Tfh), and Th17 cells were discerned based on surface marker expression patterns. Additionally, intracellular cytokine staining was performed for interferon-γ, interleukin (IL)-4, IL-21, and IL-17 and serum levels of these cytokines were analyzed.

Results

In patients with pSS, frequencies of circulating Tfh cells and Th17 cells were increased at baseline compared with HC, whereas frequencies of Th1 and Th2 cells were unchanged. B cell depletion therapy resulted in a pronounced decrease in circulating Tfh cells, whereas Th17 cells were only slightly lowered. Frequencies of IL-21-producing and IL-17-producing $CD4^+$ T cells and serum levels of IL-21 and IL-17 were also reduced. Importantly, the decrease in circulating Tfh cells was associated with lower systemic disease activity over time, as measured by the European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index scores and serum IgG levels.

Conclusion

B cell depletion therapy in patients with pSS results in normalization of the elevated levels of circulating Tfh cells. This reduction is associated with improved objective clinical disease activity measures. Our observations illustrate the pivotal role of the crosstalk between B cells and Tfh cells in the pathogenesis of pSS.

INTRODUCTION

To date, the immunopathogenetic mechanism of primary Sjögren's syndrome (pSS) has not been fully elucidated. Both T cells (predominantly $CD4^+$ T cells) and B cells infiltrate the salivary glands. This inflammatory response is accompanied by loss of glandular architecture and impaired function of the gland [1]. B cell hyperactivity is a prominent feature of the disease, which is manifested by hyperimmunoglobulinemia, the presence of anti-SSA/Ro and anti-SSB/La autoantibodies, and the significantly increased risk for development of mucosa-associated lymphoid tissue B cell lymphoma [2]. B cells are therefore thought to play a critical role in immunopathogenesis of pSS.

In addition to B cells, different $CD4^+$ T cell subsets may be involved in pSS pathogenesis. Th1 cells and Th17 cells seem to infiltrate the glands at an early stage of disease, as evidenced by detection of interferon (IFN)-γ and interleukin (IL)-17, whereas infiltration of follicular Th (Tfh) cells is associated with later stages [3]. Tfh cells are involved in formation of germinal centers (GC) and generation of (autoreactive) plasma cells [4]. Because the frequency of circulating Tfh cells is increased in patients with pSS and given their role in B cell activation, Tfh cells may play a prominent role in the establishment and maintenance of B cell hyperactivity in pSS [5,6].

B cell hyperactivity may be targeted by B cell depletion therapy with rituximab (RTX), an anti-CD20 monoclonal antibody. RTX seems a beneficial treatment modality for pSS patients with moderate to high systemic disease activity [7–10], although this was not confirmed in a large randomized trial, nor in patients with longstanding disease [11,12]. It is therefore important to understand how and in which subgroup of patients RTX treatment may improve clinical outcome. B cell depletion therapy with RTX results in only a moderate drop in serum IgG levels [8,11,13], while long-lived IgA- or IgG-producing cells persist in the parotid glands [14]. Thus, also B cell functions other than antibody production are probably implicated in the mode of action of B cell depletion therapy in pSS. These functions may comprise antigen presentation and secretion of various regulatory and proinflammatory cytokines [15,16]. Cytokine secretion by B cells may affect the differentiation of naive $CD4^+$ T cells into effector $CD4^+$ T cell subsets. In turn, $CD4^+$ T cells may subsequently influence the activity of B cells [17,18]. We hypothesize that this crosstalk plays a critical role in the pathogenesis of pSS. To test this hypothesis, we assessed the effect of B cell depletion therapy with RTX on the distribution and *in vitro* cytokine-producing capacity of circulating effector $CD4^+$ T cell subsets in patients with pSS and its relation to objective measures of disease activity.

MATERIALS AND METHODS

Study population and procedures

For our present study, patient material was used from our previously reported open-label study on RTX, which was an extension of our placebo-controlled study [8]. The extension study included 28 patients with pSS [13]. Twenty-four patients were analyzed in the current study; 4 patients were not included because of unavailability of stored peripheral blood mononuclear cell (PBMC) samples (n = 3) or serum sickness-like manifestations after the first dose of RTX (n = 1). All patients fulfilled the revised American-European Consensus Group criteria for pSS [19]. Eighteen of these 24 patients were treated before with RTX during previous studies and received their second (n = 13), third (n = 3), or fourth (n = 2) course of treatment; the remaining 6 patients were RTX-naive. For the patients who were re-treated with RTX, re-treatment started at least 1 year after the last course, i.e. after reappearance of circulating B cells to baseline levels and after recurrence of symptoms. Parotid gland biopsies were available from all patients before treatment and in addition from 3 patients at 16 weeks after treatment. Patient characteristics at baseline are presented in Table 1. Patients were treated on days 1 and 15 with intravenous (IV) injections of 1000 mg RTX (Roche). To minimize the risk of infusion reactions and serum sickness, all patients were treated with methylprednisolone (100 mg IV), acetaminophen (1000 mg orally) and clemastine (2 mg IV) prior to each infusion and received oral prednisone for 5 days after each infusion. Except for these precautionary medications, treatment with concomitant immunosuppressants was discontinued, including hydroxychloroquine and oral prednisone.

PBMC were isolated at baseline and 16, 24, 36, and 48 weeks after the first dose of RTX in the current study design. At the same time points, objective signs of disease activity were measured, including the European League Against Rheumatism (EULAR) Sjögren's syndrome Disease Activity Index (ESSDAI) [20], ESSDAI without the biological domain (ClinESSDAI) [21], serum IgG levels, and stimulated whole saliva production. Twenty-four age- and sex-matched healthy individuals were included in the study as controls (HC). All patients and HC provided informed consent and the study was approved by the local Medical Ethics Committee of the University Medical Center Groningen (METc2008.179).

Analysis of effector CD4$^+$ T cell subsets by flow cytometry: surface marker analysis

Effector CD4$^+$ T cell subsets can be further divided into 4 subsets by using a combination of different chemokine receptors [22,23] Accordingly, we defined Th1, Th2, Th17, and Tfh cells as CD45RA$^-$FoxP3$^-$CXCR5$^-$CXCR3$^+$CCR4$^-$CCR6$^-$, CD45RA$^-$FoxP3$^-$CXCR5$^-$CXCR3$^-$CCR4$^+$CCR6$^-$, CD45RA$^-$FoxP3$^-$CXCR5$^-$CXCR3$^-$CCR4$^+$CCR6$^+$ and CD45RA$^-$FoxP3$^-$CXCR5$^+$PD-1$^+$ CD4$^+$ T cells, respectively (described in Supplementary Figure 1). For flow cytometry

analysis, cryopreserved PBMC from 24 patients with pSS and 24 HC were stained for surface markers with antibodies listed in Supplementary Table 1. Subsequently, cells were stained with a fixable viability dye (eBioscience), fixed and permeabilized in Fix/Perm buffer and stained with anti-FoxP3 (eBioscience). Fluorescence-minus-one control stains were included to determine background levels of staining. After staining, cells were washed and immediately analyzed by flow cytometry.

In vitro cytokine production analysis

In addition to surface marker analysis, *in vitro* stimulation and intracellular cytokine staining was performed using PBMC from 20 consecutive patients with pSS and 11 consecutive HC. Cryopreserved PBMC were thawed and stimulated *in vitro* with 5 ng/mL phorbol myristate acetate (Sigma-Aldrich) and 0,17 µg/mL calcium ionophore A23187 (Sigma-Aldrich) for 16 h at 37°C and 5% CO_2. These conditions were tested as optimal for analysis of *in vitro* cytokine production by T cells after thawing. As a negative control, 1 sample of each cell suspension remained without stimulation. Directly after addition of the stimulants, 10 µg/mL brefeldin A (Sigma-Aldrich) was added. After stimulation, cells were fixed and permeabilized in Fix/Perm buffer (eBioscience) for 45 minutes at 4°C. Then, samples were labeled by fluorescent cell barcoding (FCB) [24]. After washing and permeabilization, different FCB-labeled samples were combined into 1 FACS-tube and stained for 1 h with antibodies listed in Supplementary Table 1. After staining, cells were washed and analyzed by flow cytometry for intracellular production of IFN-γ, IL-4, IL-17, and IL-21, which are 4 signature cytokines of Th1, Th2, Th17, and Tfh cells, respectively. Because stimulation reduces surface expression of CD4 on T cells, cytokine-producing $CD4^+$ T cells were identified indirectly by gating on $CD3^+CD8^-$ lymphocytes. Unstimulated samples were used to delineate positive and negative populations and results are expressed as the percentage of single cytokine-producing cells within the total $CD4^+$ T cell population. Dead cells not washed out by several washing steps were excluded on the basis of their forward- and side-scatter profile.

For all flow cytometry analyses, data were acquired using a FACS-LSRII flow cytometer (Becton Dickinson) and analyzed using the Kaluza 1.2 Flow Analysis software (Beckman Coulter) or FlowJo analysis software (TreeStar).

Absolute cell count in blood samples

In patients with pSS, absolute numbers of circulating $CD4^+$ T cells and $CD19^+$ B cells were measured in fresh blood samples by using the MultiTest TruCount method (Becton Dickinson), as described by the manufacturer. These numbers were used to convert the frequencies of different $CD4^+$ T cell subsets into absolute numbers.

Measurement of serum cytokines and autoantibodies

Serum levels of IL-21, IL-17, IFN-γ, and IL-4 were measured using a ProcartaPlex human cytokine immunoassay (eBioscience), according to the manufacturer's protocol. Serum levels of anti-SSA/Ro and anti–SSB/La at baseline and week 16 were measured with a Phadia 250 analyzer using EliA Ro and EliA La (Phadia). Levels were expressed in arbitrary units per milliliter (AU/mL).

Quantitative PCR and immunofluorescence for detection of glandular IL-21 expression

Total RNA was extracted from microdissected infiltrated regions of frozen parotid gland tissue, using the RNeasy Micro Kit (Qiagen). cDNA was subsequently synthesized. Primers for β-actin (forward 5′-GAG CGG GAA ATC GTG CGT GAC-3′ and backward 5′-AGG AAG GAA GGC TGG AAG AGT GC-3′) and IL-21 (forward 5′-AAG CTC CCA AGG TCA AGA TCG-3′ and backward 5′-AGC AGG AAA AAG CTG ACC ACT-3′) were used for SYBR Green-based real-time quantitative PCR analysis (Bio-Rad). The mean of triplicate results was normalized to expression of endogenous transcripts encoding for β-actin to calculate relative mRNA expression of IL-21. Fold change values were determined using the 2^(ΔΔCt) method. The median baseline cycle threshold (Ct) value was used as calibrator. For analysis of IL-21 protein expression, formalin-fixed, paraffin-embedded sections (4 μm) of parotid gland tissue were incubated with primary antibody to IL-21 overnight at 4°C (NBP1-02706, Novus Biologicals). Prior to incubation, heat-induced epitope retrieval was performed using citrate buffer (pH 6). Anti-IL-21 antibodies were visualized using anti-rabbit-Af594-conjugated antibody (Invitrogen).

Statistical analysis

Statistical analysis was performed using SPSS Statistics 20 software (IBM). Results are presented as mean ± standard deviation (SD) or median (interquartile range), for normally and non-normally distributed data, respectively. The Mann-Whitney U test was used to compare differences in frequencies of effector $CD4^+$ T cell subsets between patients and HC. Generalized estimating equations (GEE) with exchangeable correlation structure were used to analyze the presence of different T cell subsets over time within subjects. If residuals were non-normally distributed, parameters were square-root or log transformed before entering into the equation. We have previously reported that B cells started to reappear at week 24 after RTX treatment [25]. Therefore, data from baseline up to week 24 were used to assess change over time compared with baseline, i.e. during B cell depletion. Data from week 24 up to week 48 were used to assess change over time compared with week 24, i.e. during B cell repopulation. Associations between the development of different objective disease variables and

T cell subsets were also tested with GEE. Two-tailed P values < 0.05 were considered statistically significant.

TABLE 1 | Baseline characteristics of patients with primary Sjögren's syndrome treated with rituximab. Results are expressed as mean ± SD or median (IQR), unless otherwise indicated.

Characteristics	Patients, n=24
Age, yrs	44 ± 14
Female gender, n (%)	23 (96)
Disease duration, mos	61 (46-110)
ESSPRI	7.0 (6.0-8.3)
ESSDAI	8 (4-11)
ClinESSDAI	7 (4-11)
IgG, g/L	21 (18-25)
SWS, mL/minute	0.31 (0.15-0.50)
Focus score	1.9 (1.4-2.9)
Presence of GC, n (%)	7 (32)
Presence of LEL, n (%)	15 (68)

Histopathological data were available from 22 patients. IQR: interquartile range; ESSPRI: European League Against Rheumatism (EULAR) Sjögren's syndrome Patient Reported Index; ESSDAI: EULAR Sjögren's syndrome Disease Activity Index; ClinESSDAI: ESSDAI without inclusion of the biological domain; IgG: total immunoglobulin level in serum; SWS: stimulated whole saliva production rate; GC: germinal centers; LELs: lymphoepithelial lesions.

RESULTS

Frequencies of circulating Tfh cells and Th17 cells, defined by surface marker expression, are increased in patients with pSS compared with HC

To study homeostasis of $CD4^+$ T cell subsets with acquired effector function in patients with pSS, we analyzed the relative distribution of $CD4^+CD45RA^-$ T cell subsets, i.e., antigen-experienced memory $CD4^+$ T cells. Analysis of surface marker expression revealed that frequencies of $CD4^+CD45RA^-FoxP3^-CXCR5^+PD\text{-}1^+$ cells were increased in patients with pSS compared with HC (Figure 1A; $P < 0.001$). These $CD4^+CD45RA^-CXCR5^+PD\text{-}1^+$ cells can be considered as circulating counterparts of Tfh cells in lymphoid tissue, because they share phenotypical and functional characteristics [26,27]. We therefore refer to them as circulating Tfh (cTfh) cells. Frequencies of Th17 cells were also increased in patients with pSS (Figure 1B; $P = 0.009$). Of note, there was a positive correlation between frequencies of cTfh and Th17 cells (Spearman's $\rho = 0.44$; $P = 0.031$). No significant differences were observed in frequencies of Th1 cells or Th2 cells between patients with pSS and HC (Supplementary Figure 2).

Analysis of effector subsets by surface marker expression does not necessarily reflect cytokine-producing capabilities of these cells. We therefore subsequently analyzed the production of IL-21, IL-17, IFN-γ, and IL-4 by *in vitro* stimulated $CD4^+$ T cells from

patients with pSS and HC. Despite the observation that frequencies of surface marker-defined cTfh cells and Th17 cells were elevated, patients with pSS did not display aberrant frequencies of IL-21-producing or IL-17-producing CD4+ T cells at baseline, compared with HC (Figure 1C-D). Also no substantial alterations in frequencies of CD4+ T cells producing IFN-γ or IL-4 were observed in patients with pSS, compared with HC (Supplementary Figure 2). For all surface marker-defined and cytokine-defined CD4+ T cell subsets, no significant differences were observed between RTX-naive patients and patients who had received RTX previously (data not shown).

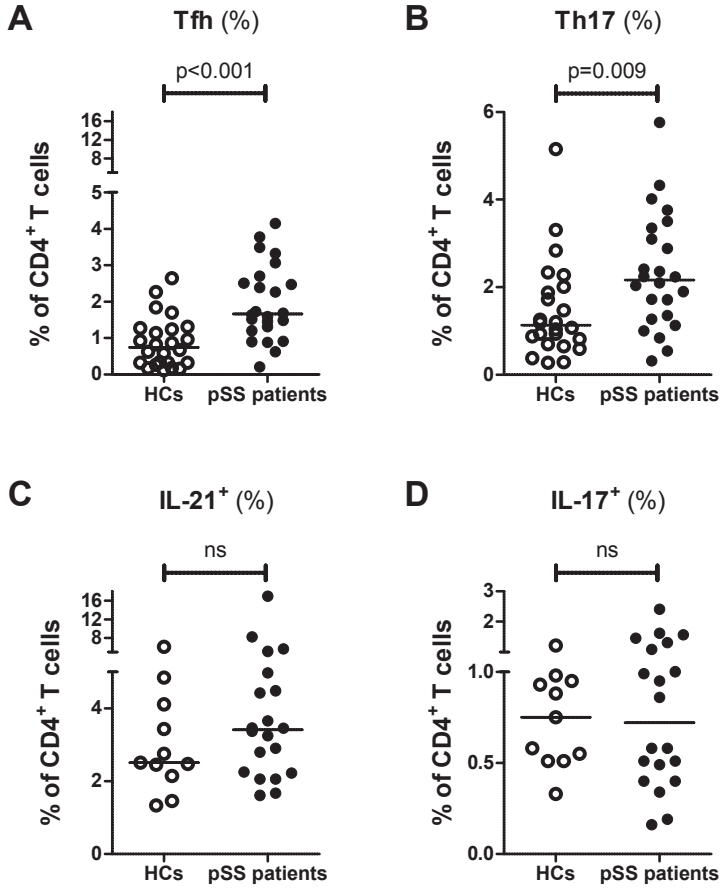

FIGURE 1 | Frequencies of Tfh cells and Th17 cells are increased at baseline in patients with pSS.

Frequencies of circulating (**A**) Tfh cells ($CD45RA^-CXCR5^+PD\text{-}1^+$) and (**B**) Th17 cells ($CD45RA^-CXCR5^-CXCR3^-CCR4^+CCR6^+$) in patients with pSS (n = 24) and HC (n = 24) are displayed. Additionally, frequencies of circulating (**C**) IL-21 and (**D**) IL-17-producing CD4+ T cells in patients with pSS (n = 20) and HC (n = 11) are displayed. Horizontal lines indicate the median. P value < 0.05 was considered statistically significant. P values were calculated using the nonparametric Mann-Whitney U-test. Tfh: follicular Th; pSS: primary Sjögren's syndrome; HC: healthy controls; IL: interleukin.

Frequencies of circulating Tfh cells (and to a small extent also Th17 cells) are reduced by B cell depletion therapy

Next, the effect of B cell depletion therapy with RTX on circulating effector CD4$^+$ T cell subsets was evaluated. B cell depletion therapy resulted in a decrease of almost 50% in frequencies of cTfh cells (Figure 2A; P < 0.001). At week 24, frequencies of cTfh cells in patients with pSS reached levels comparable to HC. Although Th17 cell frequencies did not decrease at the population level, a small but significant decrease in individual patients over time was observed by GEE analysis (Figure 2B; P = 0.041). No changes in frequencies of Th1 and Th2 cells were observed (Supplementary Figure 3). During B cell repopulation, frequencies of cTfh cells returned to baseline levels of the patients (Figure 2A; P = 0.042). Analysis of absolute numbers of effector CD4$^+$ T cell subsets during B cell depletion revealed that cTfh cells decreased significantly, whereas Th17, Th1, and Th2 cells were not significantly altered (Supplementary Table 2).

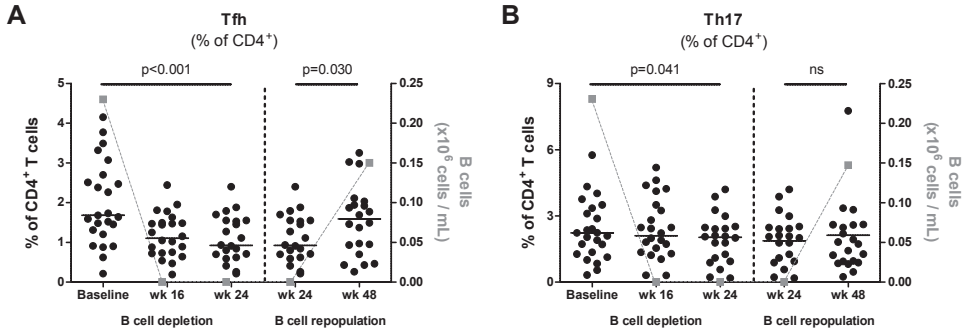

FIGURE 2 | Predominantly Tfh cells decrease after rituximab treatment. Frequencies are displayed of circulating (**A**) Tfh cells (CD45RA$^-$CXCR5$^+$PD-1$^+$) and (**B**) Th17 cells (CD45RA$^-$CXCR5$^-$CXCR3$^-$CCR4$^+$CCR6$^+$) during B cell depletion (week 0-24) and B cell repopulation (week 24-48). Horizontal lines (black) indicate the median. Median blood CD19$^+$ B cell numbers are shown in gray. The development of frequencies within subjects over time during B cell depletion was analyzed using generalized estimating equations. Frequencies within subjects during B cell repopulation were analyzed using the Wilcoxon matched pairs test. P value < 0.05 was considered statistically significant. Tfh: follicular Th.

Frequencies of IL-21-producing and IL-17-producing CD4$^+$ T cells are also reduced by B cell depletion therapy

Although frequencies of both IL-21-producing and IL-17-producing CD4$^+$ T cells were not significantly altered in pSS patients at baseline, compared with HC, these cytokine-producing cells were significantly affected by B cell depletion therapy (Figure 3A). Both frequencies of IL-21-producing CD4$^+$ T cells (Figure 3B; P = 0.011) and IL-17-producing CD4$^+$ T cells (Figure 3B; P < 0.001) were reduced during B cell depletion. In contrast, no substantial alterations in frequencies of circulating CD4$^+$ T cells producing IFN-γ or

IL-4 were observed (Supplementary Figure 3). During B cell repopulation, frequencies of IL-21-producing and IL-17-producing $CD4^+$ T cells increased again to levels present at baseline (Figures 3B). Analysis of absolute numbers of $CD4^+$ T cells producing IL-17 during B cell depletion and repopulation gave comparable results. Absolute numbers of IL-21-producing $CD4^+$ T cells were not significantly affected by B cell depletion, although a trend towards lower numbers was present (Supplementary Table 2).

Serum IL-21 and anti-SSA/-SSB levels are lowered by B cell depletion therapy

Because we observed that cTfh cells were reduced by B cell depletion therapy, and given the observation that treatment results in lowering of total IgG levels (± 15%) [13], we analyzed whether serum levels of IL-21, anti-SSA/Ro, and anti-SSB/La were also affected. Serum IL-21 was significantly lowered by B cell depletion (Supplementary Figure 4; $P < 0.001$). Importantly, decrease in serum IL-21 was associated with the reduction in cTfh cells over time ($P < 0.001$). Serum levels of IL-17, IFN-γ, and IL-4 were detectable at low levels in 58%, 92% and 42% of the patients at baseline, respectively. During B cell depletion, in patients with detectable levels at baseline, a decrease in IL-17 and IL-4 was observed, whereas IFN-γ levels remained unchanged (Supplementary Figure 4).

Anti-SSA/Ro titers were decreased by about 25% at week 16, compared with baseline [8498 (5989-13009) AU/mL at week 16; 11312 (7196-18254) AU/mL at baseline; $n = 24$; $P < 0.001$]. A similar reduction of ± 25% was observed for anti-SSB/La titers [249 (80-4966) AU/mL at week 16; 339 (140-6886) AU/mL at baseline; $n=18$; $p<0.001$]. In addition to serum levels of IL-21, mRNA and protein expression levels of IL-21 were analyzed in parotid gland tissue of 3 patients at baseline and 16 weeks after treatment. Both mRNA and protein expression tended to decrease after treatment (Supplementary Figure 5). These data suggest that not only numbers of cTfh cells are affected by B cell depletion therapy, but also their functionality in terms of IL-21 production, likely also locally in the inflamed salivary glands, resulting in less B cell help.

Reduced frequencies of circulating Tfh cells and Th17 cells are associated with improvement in objective measures of disease activity and autoantibody titers

Because cTfh and Th17 cells, as well as IL-21-producing and IL-17-producing $CD4^+$ T cells were affected by B cell depletion therapy, we subsequently analyzed associations between changes in these subsets and objective measures of disease activity over time (Table 2). Importantly, the decrease in frequencies of cTfh cells was significantly associated with amelioration of systemic disease activity, as measured by ESSDAI ($P < 0.001$) and ClinESSDAI ($P = 0.001$), and with a decrease in total IgG ($P < 0.001$), anti-SSA/Ro ($P < 0.001$) and anti-SSB/La ($P < 0.001$) in serum. Reduced frequencies of IL-21-

producing CD4+ T cells were associated only with improvement in ESSDAI (P < 0.001). The course of cTfh cells or IL-21-producing CD4+ T cells during B cell depletion did not significantly differ between patients with (n = 7) and without (n = 15) GC in parotid gland tissue at baseline. Frequencies of IL-17-producing CD4+ T cells were significantly associated with levels of IgG (P = 0.001) and anti-SSB/La (P < 0.001) over time. Further, we found that lower Th17 cell frequencies were associated with higher stimulated whole saliva production within patients over time (P < 0.001). cTfh cells tended to be associated with stimulated saliva production as well (P = 0.007).

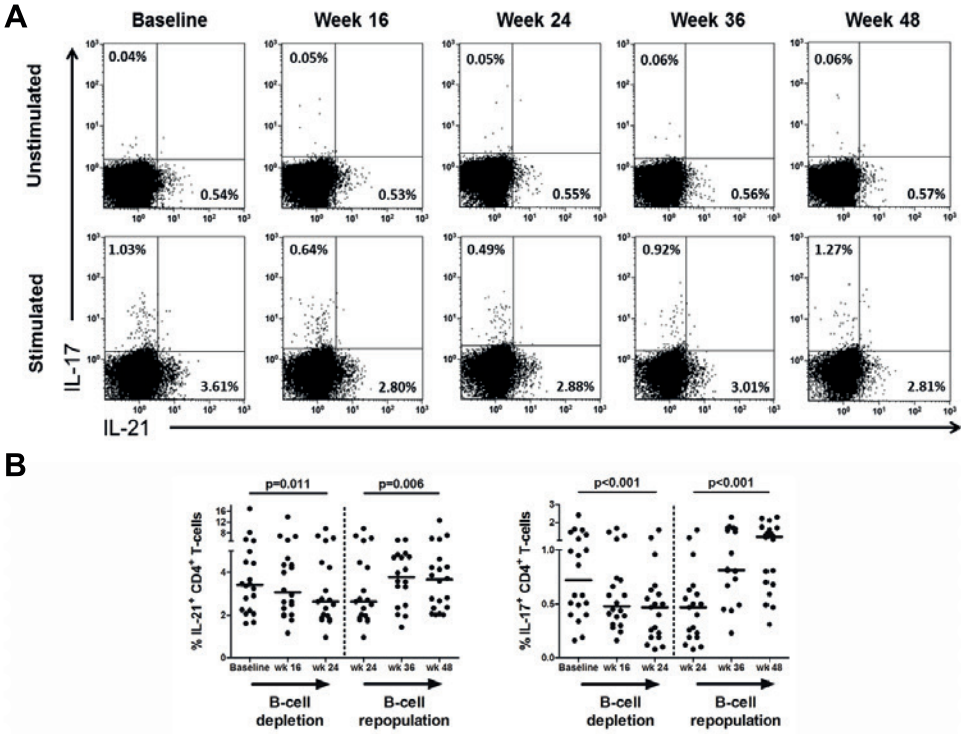

FIGURE 3 | In vitro IL-21 and IL-17 production by CD4+ T cells after rituximab (RTX) treatment.

A. Representative flow cytometry plots of *in vitro* IL-21 and IL-17 production in circulating CD4+ T cells from 1 patient with pSS at baseline and at 16, 24, 36, and 48 weeks after the first dose of RTX. The plotted cells were gated as $CD3^+CD8^-$ T cells and gate settings were based on the unstimulated sample (biological control). Values in each gate represent the percentages of positive cells. **B**. *In vitro* cytokine production by CD4+ T cells in 20 patients with pSS. Frequencies of IL-21-producing and IL-17-producing CD4+ T cells are displayed. Frequencies within subjects over time were analyzed with generalized estimating equations during B cell depletion (week 0-24) and B cell repopulation (week 24-48). Horizontal lines indicate the median. P value < 0.05 was considered statistically significant. IL: interleukin; pSS: primary Sjögren's syndrome.

TABLE 2 | Associations between clinical or laboratory parameters and effector CD4+ T-cell subsets within patients during B-cell depletion.

Objective variables	Tfh %		Th17 %		IL-21 %		IL-17 %	
	β	P	β	P	β	P	β	P
ESSDAI[a]	0.526	**<0.001**	0.204	0.021	0.131	**<0.001**	0.405	0.255
ClinESSDAI[a]	0.445	**0.001**	0.116	0.225	0.141	0.005	0.195	0.572
IgG[b]	0.084	**<0.001**	0.028	0.019	0.003	0.598	0.115	**0.001**
anti-SSA[a]	10.4	**<0.001**	2.82	0.426	-0.493	0.805	11.2	0.043
anti-SSB[b]	0.127	**<0.001**	-0.019	0.642	-0.027	0.449	0.196	**<0.001**
SWS[b]	-0.149	0.007	-0.148	**<0.001**	0.0328	0.008	-0.193	0.030

For ESSDAI, ClinESSDAI, IgG, and SWS, values from pSS patients at baseline, week 16 and week 24 were included in the generalized estimating equation (GEE) models. For anti-SSA and anti-SSB, baseline and week 16 were included in the GEE model. P values < 0.005 (Bonferroni-corrected α) were considered statistically significant and are shown in bold face. [a] Square root transformation. [b] Logarithmic transformation. Tfh: follicular Th cells; IL: interleukin; ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome Disease Activity Index; ClinESSDAI: ESSDAI without inclusion of the biological domain; IgG: serum IgG levels (g/L); anti-SSA/SSB: serum anti-SSA/SSB levels (arbitrary units/mL); SWS: stimulated whole saliva production rate (mL/min).

DISCUSSION

Here, we show by surface marker analysis that frequencies of cTfh cells ($CD4^+CD45RA^-FoxP3^-CXCR5^+PD\text{-}1^+$) and Th17 cells ($CD4^+CD45RA^-FoxP3^-CXCR5^-CXCR3^-CCR4^+CCR6^+$) are increased in patients with pSS compared with HC, while Th1 and Th2 cell frequencies are unchanged. We further show that B cell depletion therapy reduces the elevated frequencies of cTfh cells and to a smaller extent also Th17 cells. In addition to the effects on surface marker-defined cTfh and Th17 cells, a specific reduction of IL-21-producing and IL-17-producing $CD4^+$ T cells is seen after B cell depletion therapy. This is further reflected by lower serum levels of IL-21 and IL-17 after treatment. When circulating B cells repopulate, the observed effects on these pathogenic T cell subsets are reversed. Importantly, reductions in cTfh cells and to a lesser extent also Th17 cells are associated with amelioration of several objective disease activity parameters.

Our findings confirm previous observations that levels of cTfh cells and Th17 cells, as defined by surface marker expression, are increased in patients with pSS, compared with HC [5,6,26,28]. We also observed that frequencies of cTfh cells and Th17 cells at baseline are correlated. This might be because differentiation pathways for Tfh cells and Th17 cells are interconnected, mediated by IL-6 and IL-21 [4,29,30]. Although we observed an increase in both cTfh cells and Th17 cells in patients with pSS at baseline, this is not reflected by a similar increase in IL-21-producing or IL-17-producing $CD4^+$ T cells. For IL-17-producing $CD4^+$ T cells, comparable levels between patients with pSS and HC were previously noted [31,32]. Apparently, at least for cTfh cells and Th17 cells, there is no clear association between surface marker expression

and signature cytokine production by these subsets in patients with pSS. The lack of correlation may be explained in 2 different ways that are not mutually exclusive. First, it is known that cytokine production is not limited to 1 subset, because not only $CD45RA^-CD4^+CXCR5^+PD\text{-}1^+$ cells, but also $CD4^+CXCR5^+$ $PD\text{-}1^-$ cells and $CD4^+CCR9^+$ T cells are able to produce IL-21 [23,33]. Accordingly, IL-17 can be produced by all $CD4^+CCR6^+$ cells, including Th17 cells ($CD45RA^-CD4^+CCR4^+CCR6^+$ T cells) [34]. Second, not all effector subsets in the circulation are yet fully capable of cytokine production, and additional signals (e.g., proinflammatory cytokines) in the inflamed tissue are necessary for these cells to become cytokine-producing effector $CD4^+$ T cells. Dependency on local cytokine signals that drive cytokine production by effector $CD4^+$ T cells has been described for IL-17-producing $CD4^+$ T cells [34]. Likely, in patients with pSS, both cTfh and Th17 cells start to produce cytokines only upon arrival in glandular tissue. Accordingly, in minor salivary glands of patients with pSS, IL-17-producing $CD4^+$ T cells are enriched in the lymphocytic infiltrate, compared with sicca controls [35]. Further, IL-21 and IL-17 are significantly upregulated in minor salivary gland tissue of pSS patients, both at protein and mRNA level [35-38]. Local production of cytokines may also underlie the reported increase in serum levels of IL-21 and IL-17 [36,38-41]. We therefore suggest that assessment of circulating effector subsets by surface marker expression provides better prognostic value, compared with measurement of *in vitro* cytokine production by circulating $CD4^+$ T cells.

Importantly, we demonstrated in this study that B cell depletion therapy reduces both the levels of cTfh cells and IL-21-producing $CD4^+$ T cells in patients with pSS. Both effects are reversed during B cell repopulation. This observation indicates that the drop in cTfh cells is a specific effect of RTX and is not, for example, due to administration of methylprednisolone, used to prevent infusion reactions. Previous studies in type 1 diabetes and granulomatosis with polyangiitis have also shown that B cell depletion therapy affects cTfh cells [42,43]. However, the immunopathology of these diseases is significantly different from pSS and these studies have not related their findings to changes in disease activity. In the current study, we observed that reduced cTfh cell frequencies in patients with pSS after B cell depletion are strongly associated with amelioration of several objective measures of disease activity, including ESSDAI, ClinESSDAI, serum IgG, anti-SSA/Ro, anti-SSB/La, and to a lesser extent also stimulated saliva production.

Because Tfh cells are important for GC formation and plasma cell differentiation [4], reduced numbers of Tfh cells may lead to less T cell-dependent B cell activation of persisting B cells in the salivary glands. We assume that the observed reduction in cTfh cells reflects the overall reduction of Tfh cell activity in the body, because others have shown that the proportion of cTfh cells correlates well with Tfh cell activity, including autoantibody production [26,27]. Preliminary data in 3 patients indicate that glandular

Tfh cell activity in patients with pSS, as reflected by the presence of IL-21-producing cells and total levels of IL-21 mRNA, is indeed decreased by B cell depletion therapy, whereas the total number of infiltrating T cells remained unaffected by treatment [44]. Reduction of Tfh cells in patients with pSS may contribute to the decline in GC activity that is observed in parotid glands of patients with pSS after B cell depletion, even though the glands still contain appreciable numbers of B cells [45]. The combination of reduced Tfh cells and depletion of $CD20^+$ plasma cell precursors likely results in decreased generation of plasmablasts and short-lived plasma cells, which may explain lowering of anti-SSA/Ro60 antibodies that are partly produced by short-lived plasma cells [44]. However, probably not all plasmablasts are affected by treatment, because Dass, *et al* have shown using high-sensitivity flow cytometry that low numbers of circulating plasmablasts can still be present after B cell depletion therapy [45]. Although we did not analyze circulating plasmablasts in the current study, the decrease in serum IgG levels and autoantibodies after treatment suggests that at least a significant fraction of (autoreactive) plasma cell precursors is affected by treatment. B cell depletion therapy thus acts as a 2-edged sword: it results in fewer B cells that can be activated in the glands and in fewer Tfh cells that can activate the persisting B cells. Also, circulating IL-17-producing $CD4^+$ T cells are reduced after treatment, and to a minimal extent also circulating Th17 cells. The reduction in circulating IL-17-producing $CD4^+$ T cells is reflected in the target tissue, because the amount of IL-17 protein in minor salivary gland tissue of patients with pSS is reduced after B cell depletion therapy [35]. Our observation that levels of circulating Th17 cells correlate with stimulated saliva production over time provides additional evidence for the pathogenic potential of Th17 cells in the glands.

The reduction in cTfh cells and Th17 cells might be explained by direct and/or indirect effects of B cell depletion. A minor fraction of IL-17-producing $CD4^+$ T cells in peripheral blood from patients with pSS expresses low levels of CD20 [47]. Binding of RTX to CD20 may cause a direct loss of these cells. In addition, indirect effects caused by the depletion of B cells may contribute to the decrease in Th17 cells, and in particular, Tfh cells. Development of Tfh cells, and to a lesser extent also Th17 cells, is dependent on the presence of IL-6 [4,30]. B cells, and more specifically, plasmablasts are potent producers of this cytokine [48]. In line with this notion we have shown that B cell depletion therapy results in decreased serum concentrations of IL-6 in patients with pSS [39]. Depletion of B cells by RTX may therefore reduce IL-6-mediated development of Tfh cells and Th17 cells. IL-6 also stimulates the secretion of IL-21 and IL-17 by these T cell subsets [4], which may explain the reduced production of IL-21-producing and IL-17-producing $CD4^+$ T cells, and lowering of IL-21 and IL-17 levels in serum. The dependency of IL-17 production on (IL-6-producing) B cells is further illustrated by studies in mice and humans, which show that specifically Th17 activation, reflected by IL-17-production, is impaired by depletion of (IL-6-producing) B cells [49,50]. Th1 and Th2 cells are not

dependent on IL-6 for their differentiation, and this may explain why these subsets are not affected by B cell depletion therapy.

Further studies are necessary to provide direct evidence that the ablation of IL-6 -producing B cells is responsible for the observed effects of B cell depletion on $CD4^+$ T cell subsets. The strong association between cTfh cells and (Clin)ESSDAI illustrates the central role of these cells in pathogenesis of pSS. We therefore hypothesize that in particular, patients with active systemic disease are likely to benefit from B cell depletion therapy. We further suggest that the crosstalk between B cells and $CD4^+$ T cells is an appropriate target for therapy in pSS.

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SUPPLEMENTARY MATERIALS

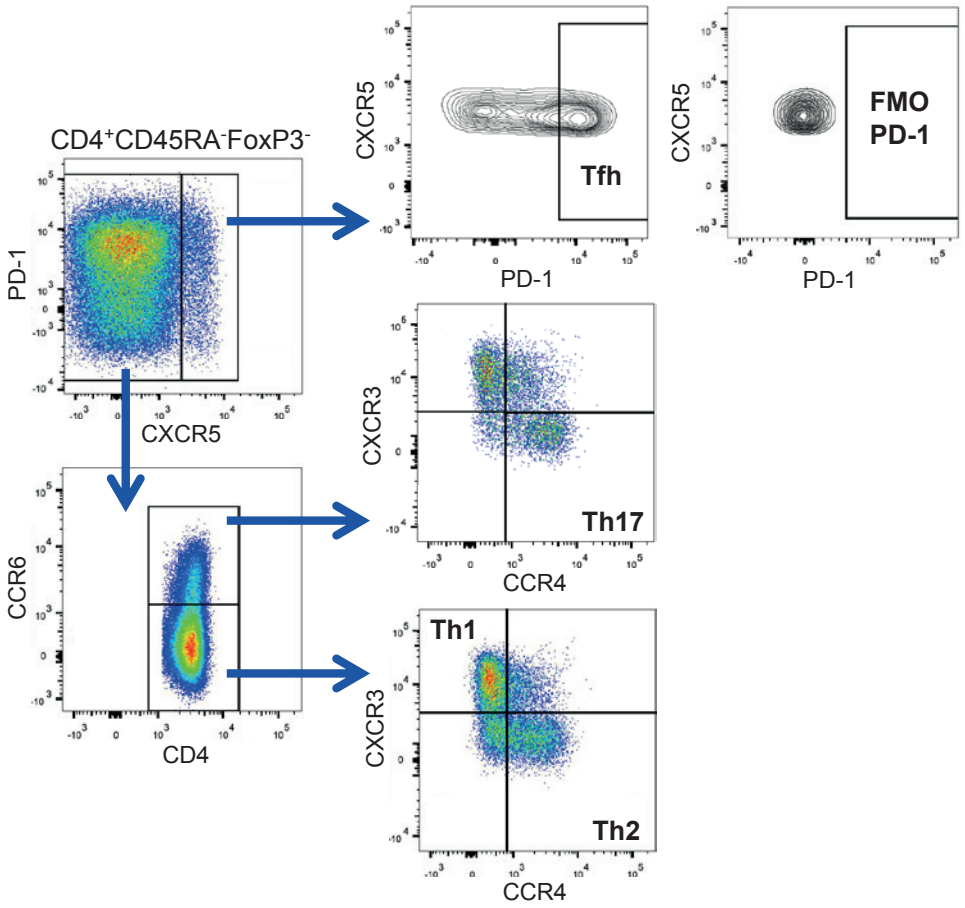
SUPPLEMENTARY TABLE 1 | Antibodies used for flow cytometry.

Antibody	Fluorochrome	Clone	Company
Surface marker expression analysis			
CD3	APC-eF780	UC1	eBioscience
CD4	AF700	OKT4	eBioscience
CD45RA	BV650	HI100	BD Biosciences
FoxP3	PE	236A/E7	eBioscience
CXCR3	BV711	1C6/CXCR3	BD Biosciences
CCR6	APC	11A9	BD Biosciences
CXCR5	PerCP-Cy5.5	RF8B2	BD Biosciences
CCR4	FITC	205410	R&D Systems
PD-1	BV786	EH12.1	BD Biosciences
Fixable viability dye	eF506	-	eBioscience
In vitro cytokine production analysis			
CD3	APC	UCHT1	BD Biosciences
CD8	PerCP	SK1	BD Biosciences
IL-21	PE	eBio3A3-N2	eBioscience
IL-17	AF488	eBio64DEC17	eBioscience
IFN-γ	AF700	B27	BD Biosciences
IL-4	PE-Cy7	MP4-25D2	Biolegend

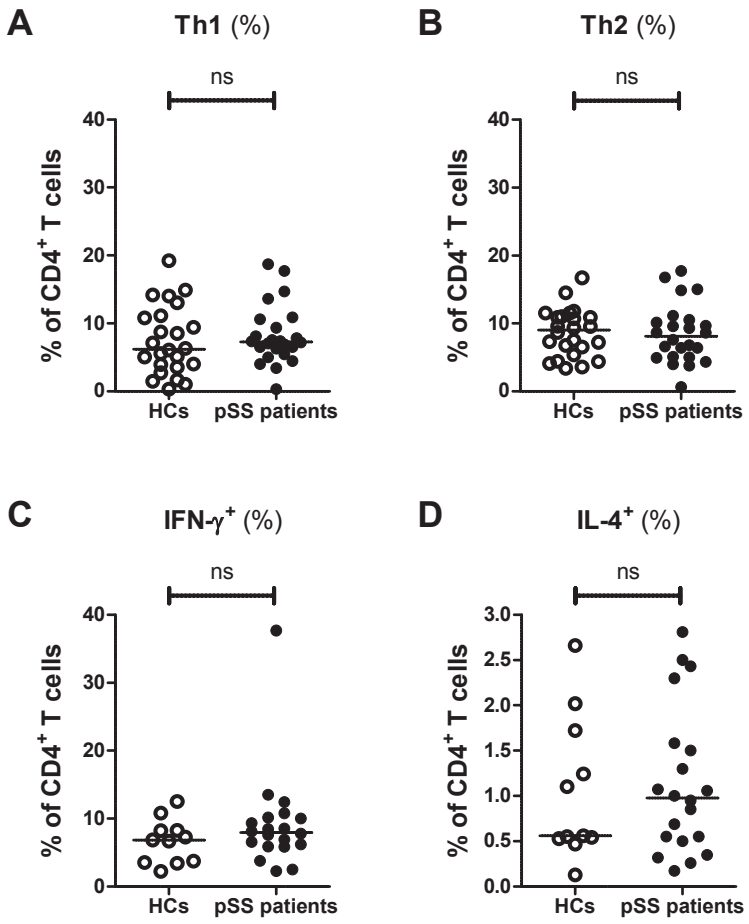
SUPPLEMENTARY TABLE 2 | Absolute numbers of different effector $CD4^+$ T cell subsets at baseline, during B cell depletion and after B cell repopulation.

	Baseline	wk 16	wk 24	P*	wk 48	P**
Tfh cells	1.02	0.64	0.84	**<0.001**	1.01	0.080
Th17 cells	1.16	1.38	1.36	0.167	1.24	0.920
Th1 cells	3.63	3.44	3.78	0.401	4.55	0.324
Th2 cells	4.44	4.49	5.94	0.253	4.83	0.825
IL-21^+ T cells	1.95	1.80	1.60	0.292	2.30	0.095
IL-17^+ T cells	0.42	0.28	0.21	**<0.001**	0.52	**<0.001**
IFN-γ^+ T cells	3.90	4.15	3.80	0.327	3.05	0.098
IL-4^+ T cells	0.40	0.52	0.36	0.769	0.48	0.205

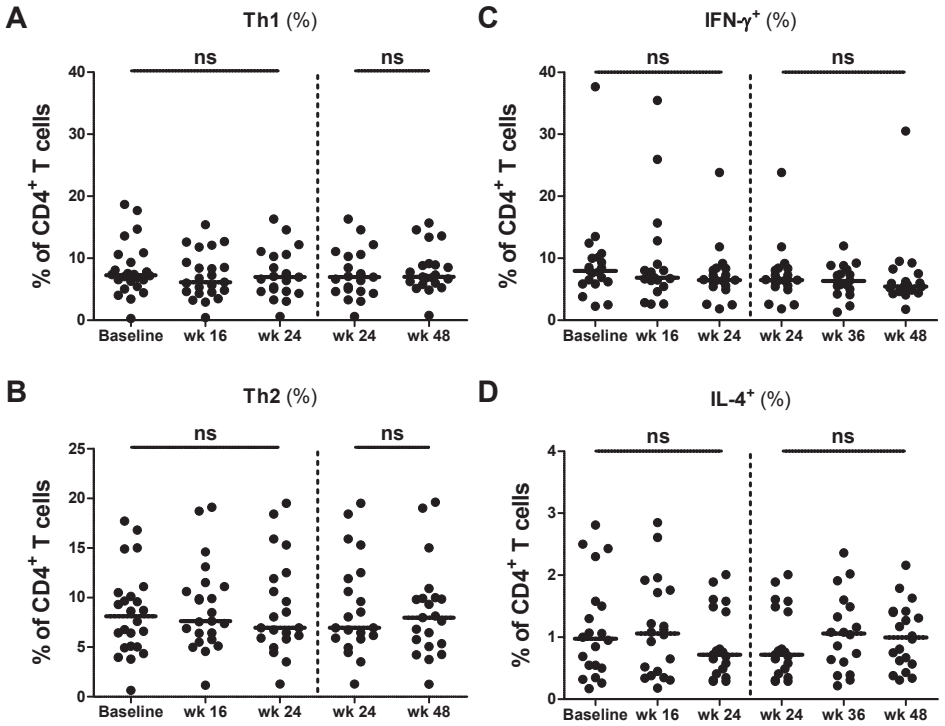
Values are presented as median absolute numbers (x10^4 cells/mL). *P value from generalized estimating equation model, including values at baseline, week 16 and week 24. **P value from Wilcoxon matched pairs test, comparing week 24 with week 48.



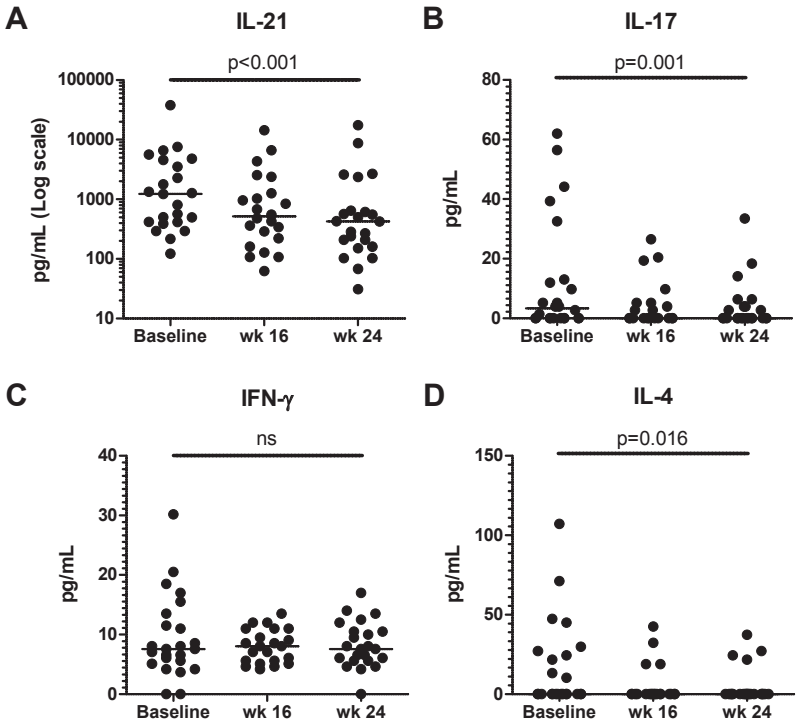
SUPPLEMENTARY FIGURE 1 | Gating strategy to discern CD4+ T cell subsets. First, memory CD4+ T cells were gated ($CD3^+CD4^+CD45RA^-FoxP3^-$). Second, Th1, Th2, Tfh, and Th17 cells were gated based on the expression pattern of surface chemokine receptors. A gating plot for the PD-1 fluorescence minus one (FMO) control is also shown. PD-1: programmed death-1.



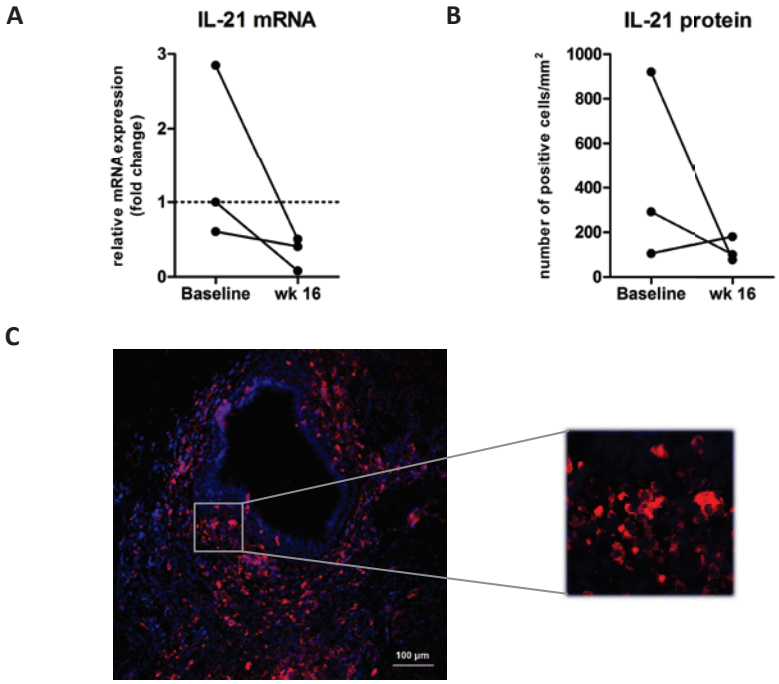
SUPPLEMENTARY FIGURE 2 | The proportion of Th1 and Th2 cells in patients with pSS at baseline compared with HC. Frequencies are displayed of circulating (**A**) Th1 cells (CD45RA-CXCR5-CXCR3+CCR4-CCR6-) and (**B**) Th2 cells (CD45RA-CXCR5-CXCR3-CCR4+CCR6-) in patients with pSS (n = 24) and HC (n = 24). Additionally, frequencies of (**C**) IFN-γ-producing and (**D**) IL-4-producing CD4+ T cells in patients with pSS (n = 20) and HC (n = 11) are displayed. Horizontal lines indicate the median. P value < 0.05 was considered statistically significant. P values were calculated using the nonparametric Mann-Whitney U-test. pSS: primary Sjögren's syndrome. HC: healthy controls. IFN: interferon. IL: interleukin.



SUPPLEMENTARY FIGURE 3 | Frequencies of Th1 cells, Th2 cells, IFN-γ-producing and IL-4-producing CD4+ T cells after RTX treatment of patients with pSS. **A**, Frequencies are displayed of Th1 cells ($CD45RA^-CXCR5^-CXCR3^+CCR4^-CCR6^-$) and (**B**) Th2 cells ($CD45RA^-CXCR5^-CXCR3^-CCR4^+CCR6^-$) (n = 24). **C**, Frequencies of IFN-γ-producing and (**D**) IL-4-producing $CD4^+$ T cells are displayed (n = 20). Values within subjects over time were analyzed with generalized estimating equations during B cell depletion (week 0-24) and B cell repopulation (week 24-48). Horizontal lines indicate the median. P value <0.05 was considered statistically significant. IFN: interferon. IL: interleukin. pSS: primary Sjögren's syndrome. RTX: rituximab.



SUPPLEMENTARY FIGURE 4 | Serum cytokines levels change after RTX treatment of patients with pSS. Serum levels of IL-21, IL-17, IFN-γ and IL-4 were measured by a multiplex bead immunoassay. Values from 24 pSS patients during B-cell depletion (week 0-24) are displayed. Changes in IL-21 and IFN-γ within patients over time were analyzed with generalized estimating equations (GEE). IL-17 and IL-4 could not be modelled using GEE and therefore values at baseline and week 24 were compared using Wilcoxon matched pairs tests. Lines indicate the median. P-value <0.05 was considered statistically significant. RTX: rituximab. pSS: primary Sjögren's syndrome. IL: interleukin. IFN: interferon.



SUPPLEMENTARY FIGURE 5 | IL-21 expression in parotid gland tissue before and after RTX. **A**, IL-21 mRNA expression levels plotted as the fold change relative to median baseline expression of IL-21 mRNA and **B**, IL-21 protein expression quantified by the number of IL-21-positive cells per mm2 of parotid gland tissue before and after B cell depletion therapy. **C**, Representative immunofluorescence image from a patient with high numbers of IL-21-positive cells in the parotid gland at baseline. Red staining represents IL-21. DAPI (blue) staining is used to label nuclei of cells.

7

THE VALUE OF RITUXIMAB TREATMENT IN PRIMARY SJÖGREN'S SYNDROME

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ABSTRACT

The rationale for B cell depletion therapy with rituximab in primary Sjögren's syndrome relies upon the well-established role of B cell hyperactivity in immunopathogenesis. In line with this notion, several biomarkers of B cell activity are significantly affected by treatment, both in the target organs and periphery. In contrast to most biological outcomes, clinical outcomes are not consistent between studies. Although two large RCTs did not meet their primary endpoint, several beneficial clinical effects of treatment have been shown. As discussed in this review, differences in study design and patient characteristics could explain the variation in results. Interestingly, a newly developed composite endpoint of subjective and objective outcomes did show a significant effect of rituximab in one of the large RCTs. Response predictors need to be identified to define more targeted inclusion criteria and achieve precision medicine. The positive effects seen on biological and clinical parameters warrant future studies to investigate this promising treatment modality.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease with a heterogeneous clinical presentation. Predominant symptoms of pSS are dryness of mouth and eyes, but many patients also suffer from extraglandular symptoms, including chronic fatigue, arthralgia and involvement of lungs, skin, kidneys and the nervous system. Dysfunction of exocrine glands is accompanied by periductal mononuclear infiltration of these glands, mainly by $CD4^+$ T cells and B cells. Involvement of B cells in pSS pathogenesis is further illustrated by the presence of autoantibodies directed against SS-A/Ro and/or SS-B /La, elevated levels of rheumatoid factor (RF), hypergammaglobulinemia, elevated levels of Bruton's tyrosine kinase in B cells and a significantly increased risk of Non-Hodgkin B cell lymphoma, predominantly mucosa-associated lymphoid tissue (MALT) lymphoma [1,2].

The prominent role of B cell hyperactivity in pSS pathogenesis provides a rationale for the use of rituximab, a humanized anti-CD20 monoclonal antibody, to treat this disease. Binding of rituximab to CD20-expressing B cells results in a significant depletion of these cells via antibody-dependent cellular cytotoxicity, complement mediated cytotoxicity and apoptosis [3]. Plasma cells are not directly depleted by rituximab, because expression of CD20 is downregulated when B cells differentiate towards plasma cells, but formation of new plasma cells may be impaired by B cell depletion therapy. Although initial studies in pSS showed improvement of both subjective and objective parameters [4-7], two large placebo-controlled trials [8,9] did not confirm all promising results of the earlier studies. Possible explanations for this discrepancy are heterogeneity in patient characteristics, primary end points and background medication use, which will all be discussed in this review. Consensus on the efficacy of rituximab in pSS is currently lacking, but treatment results in several clinical, biological and histological improvements. Furthermore, treatment studies with rituximab in pSS provided insights in the pathogenic mechanisms of the disease and post-hoc analyses of biological parameters have identified possible biomarkers that can predict response. These biomarkers may characterize subgroups of pSS patients that benefit from rituximab before start of treatment. Future studies with B cell-targeting therapy can contribute to identification of new predictors of response, as well as development of sensitive and accurate outcome measures for future clinical trials in pSS.

Effects of rituximab on B cell hyperactivity

Systemic markers of B cell hyperactivity

Several biomarkers of B cell activation, including gammaglobulins, autoantibodies (RF, anti-SS-A/Ro, anti-SS-B/La), β2-microglobulin, free light chains and B cell activating factor (BAFF/Blys) have been studied in the context of rituximab treatment in pSS (figure 1). A small but significant gradual decrease in total serum IgG after 24 weeks of treatment is seen in larger studies (Table 1) [4,8,10]. At the same time, a decrease in RF levels (up to 50%) is observed (Table 1) [4,6,10-12]. Interestingly, Dass et al. found that a non-responder had less reduction in RF after treatment compared with responders [6]. Following B cell repopulation, RF levels rise again and this rise can predict relapse of clinical symptoms [5,12]. Similar to findings in rheumatoid arthritis (RA), combined presence of RF and disease–specific autoantibodies (anti-SS-A/Ro, anti-SS-B/La) may result in higher disease activity in pSS as well [13]. The mechanism behind this synergistic effect in unknown, but crosslinking and/or stabilization of immune complexes, consisting of autoantigens and autoantibodies, by RF is likely involved. In combination with the finding that higher RF levels seem to increase the risk of lymphoma [14], these data suggest that lowering RF levels by rituximab treatment is of clinical importance, as it may protect against disease progression and/or lymphoma development in pSS.

Several studies assessed the effect of rituximab on anti-SS-A/Ro or anti-SSB/La serum levels in pSS patients. While three studies did not find significant changes in anti-SS-A/Ro or anti-SS-B/La autoantibodies after treatment [7,15,16], we found a significant reduction of ±25% in anti-SS-A/Ro and anti-SS-B/La titers at 16 weeks after treatment (Table 1) [17]. The discrepancy between studies may be explained by differences in study population size, baseline systemic disease activity, time point of measurements, or differences in reliability of the immunoassay, but methods for anti-SS-A/Ro and anti-SS-B/La measurement were not specified in most studies. The observed reduction in autoantibodies is likely a result of decreased generation of short-lived plasma cells, due to depletion of $CD20^+$ precursor cells, and/or direct depletion of CD20-expressing (short-lived) plasma cells. There is evidence that anti-SSA/Ro60 antibody production depends –at least partially- on clonal turnover of short-lived plasma cells and this may also be true for other autoantibodies [18]. B cell depletion therapy can therefore directly affect autoantibody production in pSS patients.

In addition to gammaglobulin and autoantibody levels, other indicators of B cell hyperactivity in pSS are also affected by rituximab treatment. β2-microglobulin levels show a 'delayed' drop at 16 weeks after treatment [8,12], which was not seen at 6 or 12 weeks after rituximab treatment [8,11]. Serum immunoglobulin free light chains (FLCs) are also affected by rituximab and decrease significantly from week 5 up to week 48 after treatment (unpublished data), in line with findings in RA [19]. Both β2-microglobulin and

FLC baseline levels in serum of pSS patients are positively correlated to ESSDAI scores [20], suggesting that there is a link between the degree of B cell activation and systemic disease activity. Serum levels of several B cell-associated cytokines, including IL-6, GM-CSF, TNF-α and IL-10, are also lowered by rituximab [21]. Whether this decrease is the consequence of removal of cytokine-producing B cells, or is caused by indirect effects of B cell depletion on cytokine production by other cells is not yet known. In contrast to the B cell-associated cytokines mentioned above, serum BAFF levels increase after B cell depletion therapy, likely due to unavailability of BAFF receptors as a consequence of the absence of B cells [22]. This rise in BAFF levels may be unfavorable for the patient due to enhanced survival of autoreactive B cell clones and skewing of newly formed B cells towards an autoreactive phenotype [23,24]. Therefore, the efficacy of therapy combining B cell depletion and BAFF-blockade is currently under investigation (NCT02631538).

In summary, most biomarkers of B cell activation in the circulation are decreased by B cell depletion therapy (figure 1). Lowering of B cell activation likely contributes to amelioration of systemic disease activity in pSS patients, due to lower levels of autoantibodies and pro-inflammatory cytokines.

TABLE 1 | Main biological effects of rituximab treatment analyzed in prospective clinical studies.

Study population	Patients treated (*n*)	IgG	RF	Anti-SSA titer	Glandular B cells	Patients on concomitant immunomodulatory drugs (*n* (%))	
						DMARDS	Steroids
Pijpe et al., 2005	15	=	↓†	NA	↓	3 (20)	3 (20)
Devauchelle-Pensec et al., 2007	16	=	=	↓‡	↓	0 (0)	0 (0)
Dass et al., 2008	8	↓‡	↓	NA	NA	NA	NA
Meijer et al., 2009	5	NA	↓	NA	NA	0 (0)	0 (0)
Meijer et al., 2010	20	↓	↓	NA	↓	0 (0)	0 (0)
Gottenberg et al., 2012	78	NA	NA	NA	NA	29 (37)	17 (22)
Carubbi et al., 2013	19	=	=	=	↓	0 (0)	19 (100)
St. Clair et al., 2013	12	NA	↓‡	=	NA	8 (67)	3 (25)
Devauchelle-Pensec et al., 2014	63	↓	NA	NA	↓	10 (19)	17 (32)
Meiners et al., 2015	28	↓	↓	↓	NA	0 (0)	0 (0)
Bowman et al., 2017	67	NA	NA	NA	NA	39 (58)	7 (10)

Arrows indicate a decrease. NA = not available. † Only in patients with MALT/pSS. ‡ Not statistically significant.

Histological markers of B cell hyperactivity

B cells infiltrate the glandular tissue of pSS patients, accumulate around the ductal epithelium and, together with stromal cells and follicular dendritic cells, orchestrate formation of ectopic lymphoid tissue. Importantly, rituximab clearly reduces the total number and proportion of infiltrating B cells in both minor and major salivary glands (Table 1) [7,25,26]. In addition, as shown in minor salivary gland tissue, rituximab decreases mRNA expression of lymphotoxin (LT)-α and –β, important for lymphoid organogenesis [7]. The reduction of lymphotoxin is likely a direct result of lower B cell numbers in the glands, as the heterodimer LTα1β2 is mainly produced by B cells [27]. Lowering of B cell numbers is further accompanied by a decline in germinal centers located within ectopic lymphoid tissue of the glands [28]. This decline is likely caused both by direct depletion of B cells, as well as reduced presence of Tfh cells (figure 1) [17]. B cells often infiltrate the ductal epithelium of the salivary glands, resulting in the development of lymphoepithelial lesions. Most of these intra-epithelial B cells belong to a unique subset of cells expressing FcRL4 and these cells possibly function as precursor cells for MALT lymphoma [29]. We have found that intra-epithelial FcRL4+ B cells are almost completely depleted by rituximab [29]. Furthermore, rituximab treatment reduces the severity of lymphoepithelial lesions, and concomitantly leads to restoration of the epithelium [25]. It would be of value to study whether rituximab-treated patients develop MALT-lymphoma less frequently than untreated patients.

As expected, plasma cells can persist in parotid glands of pSS patients despite B cell depletion therapy, since they lack expression of CD20 [30]. However, it is not known if absolute numbers of plasma cells in salivary glands are affected by rituximab and whether this is associated with response to treatment. In synovial tissue of RA patients, a larger decrease in synovial plasma cells was observed in responders versus non-responders [31]. Therefore, it is of interest to study local plasma cell numbers in pSS patients after rituximab.

Effects of rituximab on the $CD4^+$ T cell compartment

Depletion of B cells abrogates antigen presentation and cytokine production by these cells and rituximab treatment may therefore affect other cell types, in particular $CD4^+$ T cells (figure 1) [32]. Patients with pSS have elevated proportions of circulating T follicular helper (cTfh), defined as $CXCR5^+PD\text{-}1^+CD45RA^-CD4^+$ cells, compared with healthy controls [17,33,34]. The B cell hyperactivity that is present in pSS patients may favor differentiation of Tfh cells through secretion of IL-6 by activated B cells in conjunction with high expression of co-stimulatory molecules (e.g., CD40, ICOS-L) [35,36]. Tfh cells subsequently activate B cells and promote germinal center formation and plasma cell formation [36], providing a positive-feedback loop. We have recently shown that cTfh

cells, and to a smaller extent also Th17 cells, are reduced by rituximab [17]. The decrease in cTfh cells correlates with lowering of ESSDAI scores, emphasizing their potential role in the disease process. Reduced frequencies and numbers of cTfh cells and Th17 cells during B cell depletion are accompanied by decreased serum levels of IL-21 and IL-17. Th17 cells in minor salivary glands are also reduced by rituximab, but the effect on local Tfh cells is not known yet [37,38]. Depletion of the small fraction of Th17 cells that co-expresses CD20 may contribute to the decrease in Th17 cells [39]. Thus, taking all the biological effects of rituximab on (T cell-mediated) B cell hyperactivity together, these findings may –at least in part- underlie beneficial clinical outcomes of rituximab in pSS patients.

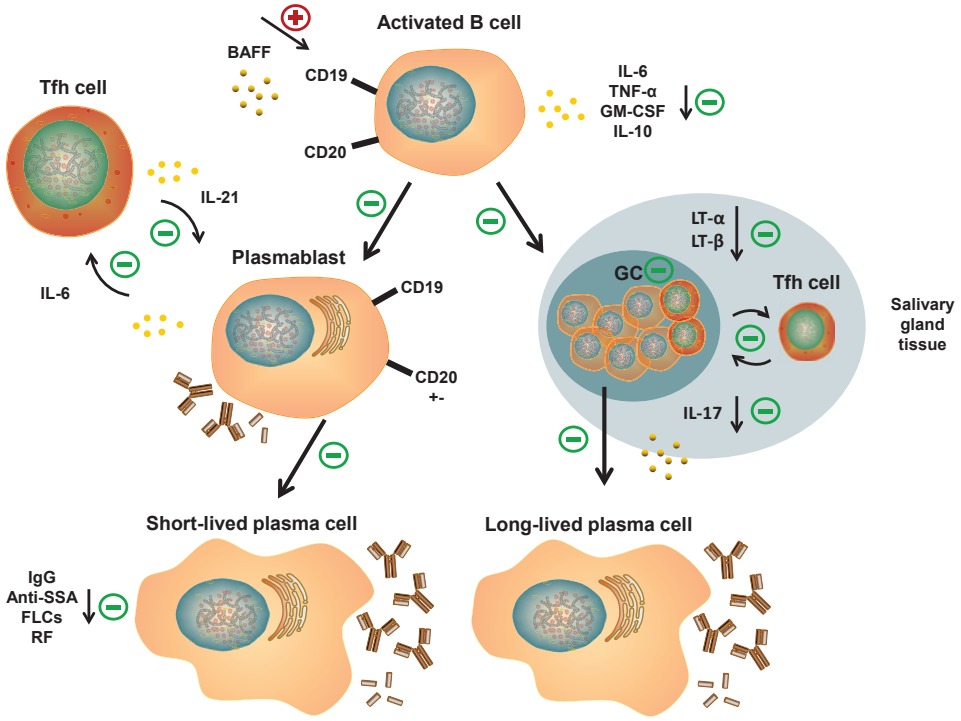

FIGURE 1 | Effects of rituximab treatment on immunopathogenesis of pSS. Cytokine production by B cells and (T cell-dependent) formation of plasmablasts and short-lived plasma cells are impaired by B cell depletion therapy with rituximab. B cell hyperactivity is reduced, as reflected by lowering of serum IgG, anti-SSA, free light chains and rheumatoid factor. Numbers of circulating Tfh cells and serum levels of IL-21 are also decreased. In salivary gland tissue, formation of ectopic lymphoid tissue and germinal centers is impaired. FLCs: free light chains, RF: rheumatoid factor.

Clinical efficacy of rituximab in Primary Sjögren's Syndrome

several open-label and randomized controlled trials have been performed to date, including two larger RCTs: the TEARS and TRACTISS trials [8,9]. In tables 2 and 3, population characteristics and clinical outcomes of all prospective clinical trials reported in literature are summarized. Despite the generally acknowledged beneficial effects of rituximab treatment on biological parameters, clinical outcomes vary between studies.

Effects on exocrine gland function and sicca symptoms

Objective measures of salivary gland function include unstimulated whole salivary flow (UWS) and stimulated whole salivary flow (SWS). UWS depends mainly on submandibular gland function, while SWS depends on both submandibular and parotid gland function. The ratio of parotid and submandibular saliva in SWS depends on the method of stimulation (mechanical vs. citric acid stimulation). UWS and SWS are both outcomes of interest. However, it is important to realize that patients show substantial intra-individual variability in salivary flow, resulting in a large standard deviation [40,41]. Therefore, adequate sample sizes are needed to show the effect of treatment on salivary gland function.

Meijer et al. and Carubbi et al. showed significant improvement in UWS after rituximab treatment [4,7]. In other trials, including the TEARS trial, no effect on UWS was observed [6,8,11,15]. Although the mean baseline UWS in the TEARS trial was comparable to the study of Meijer et al., the standard deviation was twice as high, which may influence the power of their analysis. St. Clair et al. did not find an effect on UWS, but included patients with low to absent UWS at baseline, who therefore may have had irreversible destruction of glandular parenchyma [16]. Recently, Bowman et al. showed that UWS of patients in the rituximab group of the TRACTISS trial remained stable, while the placebo group deteriorated [9].

Only few studies measured the effect of rituximab on SWS. Pijpe et al. showed that rituximab improved stimulated submandibular/sublingual salivary flow only in patients with residual salivary gland function at baseline (SWS >0.10 ml/minute) [11]. Similarly, in the RCT by Meijer et al. only patients with a SWS ≥0.15 ml/minute were included, and SWS was significantly increased in the rituximab group, while it deteriorated in the placebo group [4]. Unfortunately, recent RCTs did not measure SWS.

Currently, there is a growing interest in salivary gland ultrasound for assessment of the salivary gland structure, as it is non-invasive and inexpensive. The first study using ultrasound showed a reduction in size of the parotid and submandibular glands after rituximab treatment [32]. In a sub-analysis of the TEARS study, parotid parenchyma echostructure improved in 50% of the rituximab-treated patients versus 7% in the placebo group, visualizing histological changes induced by rituximab [33].

In summary, there seems to be a beneficial effect of rituximab on salivary gland function and structure, but the effect size is small and varies between studies. Echostructure of the gland seems to improve by rituximab, in line with the histological effects. The observed decrease in glandular B cells and (partial) restoration of the ductal epithelium in patients after treatment may contribute to the increase in salivary flow, but additional factors that affect salivary flow in pSS patients need to be identified. Lastly, it should be considered that severe destruction of parenchyma may not be reversed by immunomodulatory treatment, but such treatment could halt further damage in patients with residual gland function.

Tear gland function was assessed by Schirmer's test in most studies. Only one out of seven studies showed significant improvement in Schirmer's test after treatment (table 3) [7]. The TEARS study showed a stable Schirmer's test result in the rituximab group, whereas the placebo group tended to deteriorate [8]. Of note, Schirmer's test may not be suitable to detect small changes over time, as it shows low to moderate reliability [42]. Measurement of the epithelial integrity of the ocular conjunctiva by rose Bengal or lissamin green, and cornea by fluorescein staining is more reliable to evaluate keratoconjunctivitis sicca [43]. Interestingly, studies using these ocular surface staining methods did show improvement after treatment [4,11]. This improvement may be caused by effects of rituximab on tear gland morphology and function, composition of tear fluid, as well as effects on the inflammatory micro-environment of the ocular surface. For example, B cell-derived IL-6 levels in tears correlate with the severity of ocular surface disease, reflected by a higher extent of ocular pain, irritation and staining [4]. More knowledge about the effect of rituximab on lacrimal gland inflammation would be valuable, and ocular surface staining should be evaluated in all clinical studies, instead of using Schirmer's test only.

Besides objective measures of dryness, patient-reported outcomes (PROs) with visual analogue scales (VAS) were used in most studies to assess subjective symptoms. Positive results on total dryness scores or subscores for oral and ocular dryness were seen in most studies (table 3). Although no decrease in dryness VAS was seen in de TRACTISS study, improvement was seen in the TEARS study [8,9]. VAS dryness scores improved significantly among patients in the rituximab group, although less than 30 mm, which was set as minimum to achieve the primary end-point. Furthermore, in a post-hoc analysis, the SS Responder Index (SSRI) was developed, which includes VAS scores for fatigue, oral dryness and ocular dryness, as well as UWS and ESR. Using this composite endpoint, the proportion of patients with a 30% improvement was significantly higher in the rituximab group, compared to the placebo group [45].

Altogether, both subjective symptoms and objective measures of dryness seem to improve or at least stabilize during rituximab treatment in most studies. These findings are in accordance with histological improvements observed in the salivary glands. A

lack of robust objective tests and the poor correlation between objective tests and symptoms in pSS may underlie the reported variation in study results [46].

Effects on extraglandular manifestations

Fatigue has a major impact on quality of life in pSS patients and is therefore an important target for treatment. However, fatigue is a complex and poorly understood feature of the disease and can only be measured subjectively [47]. Most studies measured fatigue by VAS, but more detailed instruments such as the multi-dimensional fatigue inventory (MFI) and the Profile of Fatigue and Discomfort (PROFAD) questionnaire were also used. Importantly, most studies show that fatigue is reduced in pSS patients. All studies, except for the TRACTISS study and a small group of patients with advanced disease and MALT lymphoma, showed a positive effect of treatment on fatigue (table 3). The largest decrease in fatigue is often seen at early time points (week 4 in Meijer et al. and week 6 in the TEARS study). This may explain why no effect was seen on fatigue in the TRACTISS study, as the first visit in this study was scheduled in week 16. Results at early time points may have been biased by initial prednisone treatment to prevent infusion reactions. However, fatigue also improved in the open-label study by Devauchelle-Pensec et al. where no initial prednisone treatment was given [15]. In summary, although the effect size is small, most studies did show improvements in fatigue. In contrast, symptoms of arthralgia and tendomyalgia do not seem to be ameliorated during rituximab treatment (table 3).

Rituximab is often used off-label to treat severe systemic manifestations of pSS. The effect of rituximab on systemic disease activity was assessed by ESSDAI in several studies, including a prospective registry study of off-label treatment with rituximab (table 3). Substantial heterogeneity exists within and between study populations regarding systemic disease activity (table 2). A significant decrease in ESSDAI score following treatment was seen in the RCT of Meijer et al, as reported by Moerman et al., as well as two open label trials and the registry study [4,7,8,48,49]. Improvement was predominantly seen in the glandular, articular, hematological and biological domains [5], possibly because these ESSDAI domains are more likely to change [50]. The efficacy of rituximab on articular involvement was also confirmed using the 28-joint disease activity score (DAS-28) [51]. Results from the registry study and extrapolation of efficacy data from other autoimmune conditions further support the use of rituximab in pSS patients with vasculitis and pulmonary involvement [48,52]. Therefore, these specified clinical settings for rituximab treatment were recently included in the clinical practice guidelines of the Sjögren's Syndrome Foundation [52]. In contrast with earlier findings, no significant effect on ESSDAI score was seen in the TEARS and TRACTISS trials [8,9]. Whereas a lack of effect in the TRACTISS study can be explained by relatively low baseline ESSDAI scores

(mean 5.3±4.7 for the rituximab group), the mean baseline score in the TEARS study was 10±7. Of note, in the TEARS study, the ESSDAI was determined retrospectively, which may influence the accuracy and reliability. Furthermore, in the TEARS study, the prevalence of baseline involvement in the domains that show the highest sensitivity to change, e.g. glandular, constitutional, articular, hematological and biological domains, was 29%, 25%, 48%, 38% and 57%, respectively [8]. These percentages are relatively low in comparison to the study by Moerman et al. [49], in which these domains were active in 70%, 5%, 80%, 55% and 85% of patients, respectively (unpublished data). Meiners et al. and Carubbi et al. also reported a higher rate of involvement of most of these domains at baseline [5,7]. In conclusion, four prospective studies have shown beneficial effects of rituximab on systemic involvement [5,7,48,49]. The lack of effect in recent trials may be explained by low systemic involvement at baseline or heterogeneity in clinical systemic involvement.

Effects on quality of life

Several studies investigated the effect of rituximab treatment on quality-of-life using the 36-Item Short Form Health Survey (SF-36). Effects of rituximab treatment were seen in several studies in different domains of the SF-36, but with a large variability between studies (table 3). Interestingly, vitality often improved by treatment. However, the TEARS and TRACTISS trials did not observe a significant effect of rituximab treatment on SF-36 scores, compared with placebo. This is consistent with findings that subjective symptoms improved only slightly (TEARS) or not at all (TRACTISS) in the rituximab group, as subjective symptoms are strong predictors of health-related quality-of-life in pSS patients [53].

TABLE 2 | Study population characteristics.

	Study design	Patients (*n*)		Age (years)	Disease duration (years)	ESSDAI	Anti-SSA and/or –SSB positive (%)	IgG (g/L)	Unstimulated salivary flow (ml/min)	Stimulated salivary flow (ml/min)
		RTX	Control							
Pijpe et al. [11] (early pSS group)	Open label	8	0	46 ± 12	2 ± 1	NA	100	19 ± 5	0.04 (0–0.19)	0.38 (0.2–1.38)
Pijpe et al. [11] (MALT/pSS group)	Open label	7	0	54 ± 10	7 ± 4	NA	100	13 ± 6	0 (0–0.5)	0.01 (0–0.47)
Devauchelle-Pensec et al. [15]	Open label	16	0	55 ± 13	13 ± 10	NA	81	20 ± 13	0.1 ± 0.1	NA
Dass et al. [6]	RCT pilot	8	9	51 (22–64)	7 (1–18)	NA	100	19 (12–29)	NA	NA
Meijer et al. [54]	Open label *Re-treatment*	5	0	†	†	†	†	†	NA	0.09 ± 0.07‡
Meijer et al. [4] and Moerman et al. [49]	RCT	20	10	43 ± 11	5 ± 4	8 (4–13)	100	23 ± 8	0.17 ± 0.19	0.70 ± 0.57
Gottenberg et al. [48]	Registry	78	0	60 (29-83)	12 (3-32)	11 (2-31)	69	NA	NA	NA
Meiners et al. [10]	Open label *Re-treatment*	28	0	43 ± 14	7 ± 4	8 ± 5	100	23 ± 7	0.16 ± 0.18	0.42 ± 0.37
Carubbi et al. [7]	Open label	19	22	40 (27-53)	1 (1-2)	20 (6-41)	NA	NA	0.08 ± 0.04	NA
St. Clair et al. [16]	Open label	12	0	51 (34-69)	8 (2-18)	NA	83	NA	0.03 (0.0–0.22)	0.05 (0.0–0.65)
Devauchelle-Pensec et al. [8]	RCT	63	57	53 ± 13	5 ± 5	10 ± 7	81	16 ± 6	0.2 (0.4)	NA
Bowman et al. [9]	RCT	67	66	54 ± 12	5 ± 5	5 ± 5	99	18 ± 7	0.08 (0.08)	NA

Results are presented as mean ± SD or median (range). For controlled studies, patient characteristics are presented for the rituximab-treated group. † See Pijpe et al., 2005. ‡ Stimulated submandibular/sublingual flow rate.

TABLE 3 | Main clinical effects of rituximab treatment in prospective clinical studies.

Study	Study design	Patients (*n*)		Follow-up (weeks)	RTX dose	Salivary gland function	Tear gland function	Dryness VAS	Fatigue	Pain	ESSDAI	ESSPRI	SF-36
		RTX	Control										
Pijpe et al. [11] (early pSS group)	Open label	8	0	12	High	UWSF = Stim SM/SL ↑	RB ↓ Schirmer =	Oral ↓ Ocular =	MFI GF ↓	SF-36 BP =	NA	NA	Physical functioning ↑ Vitality ↑ Health change ↑ Other domains =
Pijpe et al. [11] (MALT+ pSS group)	Open label	7	0	12	High	UWSF = Stim SM/SL =*	RB ↓ Schirmer =	Oral = Ocular =	MFI GF =	SF-36 BP =	NA	NA	All domains =
Devauchelle-Pensec et al. [15,55]	Open label	16	0	36	Low	UWSF =	Schirmer =	↓	VAS ↓	VAS ↓	NA	NA	All domains except physical functioning ↑ MCS ↑ PCS ↑
Dass et al. [6]	RCT pilot	8	9	26	High	UWSF =	Schirmer =	NA	VAS ↓† PROFAD-SSI ↓†	VAS =	NA	NA	Social functioning ↑§ MCS =‡ PCS =
Meijer et al. [54]	Open label *Re-treatment*	5	0	48	High	SWSF ↑	NA	Oral ↓	MFI GF ↓	NA	NA	NA	Physical functioning ↑
Meijer et al. [4] and Moerman et al. [49]	RCT	20	10	48	High	UWSF ↑† SWSF ↑‡§	LG ↓† Schirmer =‡	Oral ↓† Ocular ↓‡§	MFI GF ↓	NA	↓§	NA	Total score ↑† Vitality ↑†
Gottenberg et al. [48]	Registry	78	0	152**	High	NA	NA	NA	NA	NA	↓	NA	NA
Meiners et al. [10]	Open label *Re-treatment*	28	0	48	High	SWSF =	NA	Oral = *** Ocular = ***	MFI GF ↓***	NA	↓	↓	NA
Carubbi et al. [7]	Open label	19	22	120	High	UWSF ↑†§	Schirmer ↑§	↓§	VAS ↓§	VAS ↓	↓§	NA	NA
St. Clair et al. [16]	Open label	12	0	52	High	UWSF = SWSF =	Schirmer =	Oral subscores ↓ Ocular =	VAS ↓	VAS =	NA	NA	Vitality ↑ MCS = PCS =
Devauchelle-Pensec et al. [8]	RCT	63	57	24	High	UWSF =	Schirmer =	↓§	VAS ↓§	VAS =	=	NA	MCS = PCS =
Bowman et al. [9]	RCT	67	66	48	High	UWSF =‡	Schirmer =	=	VAS = PROFAD-SSI =	VAS =	=	=	All domains =

High-dose: 375 mg/m2/week (4x) or 1000 mg/m^2/two weekly (2x). Low-dose: 375 mg/m2/week (2x).

Green arrows indicate significant improvements. *Stim SM/SL improved in patients with baseline SWSF >0.1 ml/minute (n=2). ** Median follow up time. *** In 15/28 patients, reported in Meiners et al., 2015 (REF). † No significant change in control group, compared to baseline. ‡Deterioration in control group, compared to baseline. § Significant difference between RTX group and control group.

RTX: rituximab. NA: not available. VAS: visual analogue scale. ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome (SS) disease activity index. ESSPRI: EULAR SS patient reported index. UWSF: Unstimulated whole salivary flow. Stim SM/SL: Stimulated submandibular/sublingual salivary flow. SWSF: Stimulated whole salivary flow. RB: Rose Bengal. LG: Lissamin Green. MFI GF: Multidimensional Fatigue Index, General Fatigue domain. PROFAD: Profile of Fatigue and Discomfort—Sicca Symptoms Inventory (PROFAD-SSI). SF-36: Short-form 36-item Health Survey. SF-36 BP: SF-36 Bodily pain domain. MCS: mental component summary score. PCS: physical component summary score.

Predictors of response to rituximab

As described in the previous section, the efficacy of rituximab varies substantially between studies. Therefore, it is important to detect possible predictors which enable selection of patients that are likely to respond to rituximab treatment. Several predictors of good clinical response to rituximab have, for example, already been identified in RA and SLE. In RA, these factors are RF or anti-CCP positivity, elevated serum IgG, low IFN activity, lower serum levels of BAFF and lower numbers of circulating plasmablasts [56]. Furthermore, the degree of B cell depletion was positively associated with clinical response in both RA and SLE [57,58]. SLE patients with a low-affinity FcγRIIIa genotype have less effective B cell depletion, as antibody-dependent cellular cytotoxicity, mediated by FcγRIIIa-positive effector cells (mostly NK cells), is impaired [59]. This genotype results in lower binding affinity of FcγRIIIa to anti-CD20 antibodies that are bound to the target B cells. Whether this FcγRIIIa genotype is also present in a subgroup of pSS patients is not known.

In pSS, some predictors of response to rituximab were evaluated. Baseline expression of B cell-related transcripts and presence of the IFN signature in blood or minor salivary glands were not associated with clinical response to rituximab in pSS [16,60]. Devauchelle-Pensec et al. did identify some candidate transcripts, but these need further validation [60]. Concerning response biomarkers in serum, lower serum BAFF levels at baseline were associated with clinical response to rituximab in pSS patients, as defined by a ≥30% improvement in at least two items of the SSRI [26]. As mentioned earlier, high BAFF levels may enhance the survival (and prevent the depletion) of autoreactive B-cell clones, residing in glandular tissue and/or bone marrow. Besides lower BAFF levels, responders to rituximab – based on the SSRI- seemed to have lower baseline B cell activity, as reflected by a significantly lower B cell proportion within the glandular infiltrate in the labial salivary glands and lower levels of serum anti-SSA and FLCs [26]. Responders also had a lower focus score (median 0.3) and a lower salivary gland ultrasonography grade at baseline, compared with non-responders [26,61]. Based on these characteristics, responders may have less irreversible gland destruction and respond to rituximab based on SSRI improvement, since VAS dryness scores and UWS are two of the five measures that constitute the SSRI.

Using a different definition of clinical response, i.e., a decrease of ≥3 in the ESSDAI, we have shown that both baseline absolute numbers of B cells and the B cell proportion in parotid gland tissue are higher in responders versus non-responders [25,62]. Explanations for the apparent discrepancy between the study of Cornec et al. and our study have been extensively discussed elsewhere [62,63]. Our findings that high absolute numbers and proportions of B cells in the parotid gland are associated with ESSDAI response suggest that the number of B cells in the target tissue influences

systemic disease activity. Likewise, the B cell proportion in the labial gland positively correlates with markers of systemic B-cell hyperactivation [64].

Together, these data indicate that rituximab may be effective in either patients with low salivary gland inflammation, to prevent further glandular damage, or in patients with high numbers of infiltrated B cells and high systemic disease activity, to ameliorate activity in specified ESSDAI domains [62].

Why does the efficacy of rituximab vary between studies?

As discussed in the previous paragraphs, results from several trials of rituximab treatment for pSS vary. First, the use of different inclusion criteria, leading to differences in baseline patient characteristics, may explain part of this variation. Since rituximab has shown to -at least- halt further deterioration of glandular function, compared with placebo, treating patients early in the disease process may prevent progression of irreversible damage to the glands. Therefore, the majority of the studies incorporated a limited disease duration (range 2-10 years) as an inclusion criterion, but still there are large differences in disease duration between the study populations. Besides disease duration, patients characteristics such as mean age, IgG levels, and salivary flow also differ among study populations. For example, mean age is ±10 years lower in the studies by Meijer et al. and Carubbi et al., and mean IgG is higher in the study by Meijer et al., compared to other studies [4,7]. In addition, there may be other unspecified patient characteristics that influence treatment response. For example, ±80% of pSS patients show poor correlation between reported ocular dryness symptoms and objective parameters of gland function, caused by either under- or over-reporting of symptoms [46]. The number of patients under- or over-reporting their symptoms included in a trial may influence the results. Moreover, a study by Lendrem et al. identified four phenotypic clusters using hierarchical clustering of patient-reported pain, fatigue, dryness, anxiety and depression, and found significant differences in IgG, lymphocytes, ESR, ESSDAI score, and UWS between groups [65]. Presumably, these groups may show different responses to rituximab treatment.

Another possible cause of discrepancies between studies is the use of (stable) background medication. In the TEARS and TRACTISS studies, respectively 51% and 68% of the patients used either concomitant DMARDs (mostly hydroxychloroquine) or prednisone (table 1). Hydroxychloroquine and prednisone both have significant effects on the immune system, making it more difficult to show additional effects of rituximab. Differences in statistical analysis may also contribute to the variation in reported outcomes. Several studies use paired tests between baseline and multiple time points, whereas specific methods for longitudinal data analysis are available that increase statistical power and reduce multiple testing problems. Generalized estimating

equations (GEE), for example, take into account the fact that repeated measurements within one individual are correlated and GEE is therefore a more powerful tool to detect even small changes over time.

Finally, discrepancy between studies is also caused by the use of different outcome measures. No consensus has been reached about the ideal combination of outcome measures to measure treatment efficacy in pSS. The two large RCTs (TEARS and TRACTISS) have used change in subjective symptoms (VAS scores) as primary outcome measures [8,9]. Subjective symptoms such as fatigue and sicca symptoms account for a great loss in quality of life and are indeed an important target for treatment. However, the sensitivity to change of these outcome measurements has not been validated, and the response goals were set quite high (30mm change in 2 out of 4 VAS scores in TEARS, 30% change of either oral dryness or fatigue VAS score in TRACTISS). These goals may have been too high, considering that the ESSPRI has a minimal clinically important improvement of 1 point (out of 10) or 15% change. Sensitivity to change may be improved by the use of more precise PROs, such as the Patient-Reported Outcomes Measurement Information System (PROMIS), developed by the National Institutes of Health [66]. Importantly, there is a poor correlation between subjective and objective measures of dryness in pSS [46]. Until we are able to understand these discrepancies, subjective and objective measurements of dryness should be equally weighted in the evaluation of treatment efficacy. In line with this notion, Cornec et al. proposed a new data-driven composite outcome which combines objective manifestations and subjective symptoms, the SSRI [45]. This outcome was established by combination of five outcome measures that were improved by rituximab in the TEARS trial. Although the combination of subjective and objective measures as primary outcome is of interest, the SSRI needs to be refined and validated in other clinical studies.

For objective measurement of systemic activity in pSS, introduction of the ESSDAI in 2010 has been a big step forward [67]. Before that, trials did not have a validated tool to assess the effect of rituximab treatment on systemic disease activity. In later trials, most improvement was seen in domains with the highest activity at baseline [5] and a minimal clinically important improvement in ESSDAI of at least three points was determined [68]. Recent trials in pSS have therefore focused on including patients with moderate-to-high ESSDAI scores (≥5), to be able to show an effect on extraglandular manifestations.

Although the ESSDAI has been validated and is now being used in most clinical trials, there are also disadvantages regarding the use of ESSDAI as outcome measure. It is now recognized that not all ESSDAI domains show sensitivity to change [50]. Consequently, even in populations with comparable mean ESSDAI scores, differences in which ESSDAI domains are active at baseline may greatly influence response to rituximab. To prove efficacy of rituximab on systemic disease activity, future trials should therefore include

patients with moderate-to-high ESSDAI scores and activity in at least one of the domains that is likely to change (biological, articular, hematological, pulmonary, and glandular domains). Prospective use of specific indices for separate domains, such as the DAS-28 for articular involvement, may provide more detailed information on efficacy. For example, it is difficult to detect moderate changes in patients with high baseline IgG levels, using the biological domain of the ESSDAI. Additionally, researchers should be aware of the complexity of ESSDAI, which needs to be completed by rheumatologists who are trained and experienced in doing so. In a multi-center setting, this may not always be the case. A more detailed user guide has been published, which may increase the accuracy of the ESSDAI [69]. Considering that rituximab shows effect in several domains of the ESSDAI, patients with high ESSDAI scores may be the target population that we should aim for. Future trials should explore composite endpoints, which include selected domains of the ESSDAI score, besides subjective symptoms and gland function.

CONCLUSIONS AND FUTURE DIRECTIONS

Rituximab shows beneficial effects on B cell activity, glandular morphology, dryness, fatigue and several extraglandular manifestations in pSS patients. Although two large RCTs did not meet their primary endpoint, the sensitivity to change of their subjective endpoints may be limited. Future trials should evaluate clinical and biological predictors of response and explore the use of composite endpoints such as the SSRI. We believe that there is still room for new trials with anti-CD20 biologicals, as well as with other B cell-targeting therapies, such as anti-CD22 or anti-BAFF/Blys antibodies for the treatment of pSS, in well-defined populations with moderate to high ESSDAI scores. At the same time, data on long-term (>1 year) efficacy of rituximab and preventive effects on development of extraglandular manifestations and/or lymphoma are needed and may support the use of rituximab in pSS. The effectiveness of pSS has not been proven for all pSS patients, but in our opinion, rituximab is of great value to treat patients with systemic manifestations of pSS and we should not throw the baby out with the bath water.

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8

ATTENUATION OF FOLLICULAR HELPER T CELL–DEPENDENT B CELL HYPERACTIVITY BY ABATACEPT TREATMENT IN PRIMARY SJÖGREN'S SYNDROME

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ABSTRACT

Objective

To assess the effect of abatacept (CTLA-4Ig), which limits T cell activation, on homeostasis of CD4+ T cell subsets and T cell-dependent B cell hyperactivity in patients with primary Sjögren's syndrome (SS).

Methods

Fifteen patients with primary SS treated with abatacept were included. Circulating CD4+ T cell and B cell subsets were analyzed by flow cytometry at baseline, during the treatment course, and after treatment was completed. CD4+ effector T cell subsets and Treg cells were identified based on expression of CD45RA, CXCR3, CCR6, CCR4, CXCR5, programmed death 1, inducible costimulator (ICOS), and FoxP3. Serum levels of anti-SSA/anti-SSB and several T cell-related cytokines were measured. Expression of ICOS and interleukin-21 (IL-21) protein was examined in parotid gland tissue at baseline and after treatment. Changes in laboratory parameters and associations with systemic disease activity (EULAR Sjögren's Syndrome Disease Activity Index [ESSDAI]) over time were analyzed using generalized estimating equations.

Results

Abatacept selectively reduced percentages and numbers of circulating follicular helper T (Tfh) cells and Treg cells. Other CD4+ effector T cell subsets were unaffected. Furthermore, expression of the activation marker ICOS by circulating CD4+ T cells and expression of ICOS protein in parotid gland tissue declined. Reduced ICOS expression on circulating Tfh cells correlated significantly with lower ESSDAI scores during treatment. Serum levels of IL-21, CXCL13, anti-SSA, and anti-SSB decreased. Among circulating B cells, plasmablasts were decreased by treatment. After cessation of treatment, all parameters gradually returned to baseline.

Conclusion

Abatacept treatment in patients with primary SS reduces circulating Tfh cell numbers and expression of the activation marker ICOS on T cells. Lower numbers of activated circulating Tfh cells contribute to attenuated Tfh cell–dependent B cell hyperactivity and may underlie the efficacy of abatacept.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by dry eyes and mouth, associated with infiltration of lacrimal and salivary glands by T and B lymphocytes [1]. Patients with pSS may also have a diversity of extraglandular manifestations such as fatigue and musculoskeletal, lung, and skin involvement.

B cell hyperactivity is a prominent feature of pSS, reflected by hypergammaglobulinemia and autoantibody production, among others [2]. Consistent with this notion, we showed that B cells from pSS patients, including antigen-inexperienced naive B cells, express elevated levels of the B cell receptor signaling molecule Bruton's tyrosine kinase (BTK) [3]. Elevated BTK levels have been linked to T cell-mediated autoimmune pathology in both rheumatoid arthritis (RA) and pSS, indicating that there is a pro-inflammatory feedback loop between B cells and $CD4^+$ T cells [3]. Although in principle, all $CD4^+$ effector T cell subsets can provide help to B-cells, T follicular helper (Tfh) cells are considered to be the most effective cells involved in T cell-dependent activation of B cells, leading to humoral immune responses [4]. Tfh cells facilitate generation of autoantibodies and formation and maintenance of germinal centers (GCs) [5]. The majority of Tfh cells are located in secondary lymphoid organs, where they characteristically express CXCR5, programmed death 1 (PD-1), inducible costimulator (ICOS), and the transcription factor Bcl-6 [5]. $CD4^+$ T cells with a similar phenotype, called circulating Tfh (cTfh) cells, have been found in peripheral blood [6]. The transcriptional profile of cTfh cells resembles that of classical Tfh cells located in lymphoid organs, except that cTfh cells do not express Bcl-6 [7]. It remains to be elucidated whether cTfh cells are precursor cells of classical Tfh cells, derive from these cells, or both [8].

Recently, Rao et al [9] showed that another $CD4^+$ T cell subset, defined as $CXCR5^-$ $PD\text{-}1^{high}$ and provisionally named peripheral helper T (Tph) cells, also promotes B cell responses and that this subset is pathologically expanded in RA patients. Like cTfh cells, Tph cells may functionally resemble classical Tfh cells. Upon activation, Tfh, cTfh, and Tph cells up-regulate costimulatory molecules essential for interaction with B cells, including PD-1 and ICOS [10,11]. In addition, all 3 subsets can secrete interleukin-21 (IL-21), which is critical for the support of B cell proliferation and differentiation [12].

Within the peripheral $CD4^+$ T cell compartment of patients with B cell-mediated autoimmune diseases, increased frequencies of cTfh cells are commonly seen [5]. In patients with pSS, proportions of cTfh cells and serum levels of their signature cytokine IL-21 are increased [6,13-15]. Tfh cells may be present in the target tissue as well, since elevated protein levels of IL-21 have been found in minor salivary glands [13]. Although Tfh cells are a major source of IL-21, we cannot exclude the possibility that other cell types that produce IL-21, such as Th17 cells, natural killer T cells, and Tph cells, are at

least partly responsible for this increase in IL-21 levels [16]. Lowering of IL-21-producing T cells may reduce T cell-dependent B cell hyperactivity in patients with pSS and is therefore a feasible treatment option.

Abatacept is a fully human fusion molecule combining cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) with IgG Fc that binds to CD80/86 and consequently impairs CD28-mediated T cell costimulation [17]. As a result, T cell-dependent B cell activation may be reduced, both indirectly by inhibiting dendritic cell-mediated differentiation of naive T cells into $CD4^+$ effector T cells and directly by blocking T cell-B cell interaction [18,19]. To date, 2 open-label studies have evaluated the efficacy of abatacept in pSS [20,21]. Adler et al [21] observed histologic, cellular, and serologic changes in response to treatment, including a decrease in local T regulatory (Treg) cells and serum gamma globulins. They also observed a small, but significant, increase in salivary gland function after treatment. The study by Meiners et al [20] showed that abatacept improves systemic disease activity and patient-reported symptoms, as measured by the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) and Patient Reported Index (ESSPRI). That study further showed that salivary gland function remained stable during treatment (24 weeks) and during follow-up after cessation of treatment (24 weeks). Although clinical effects seem promising, the effects of abatacept on human T cells in vivo and on T cell-dependent B cell hyperactivity in pSS remain elusive. Therefore, the aim of this study was to assess the effect of abatacept on the phenotypic distribution of circulating $CD4^+$ T cell and B cell subsets as well as on T cell-dependent B cell hyperactivity. We show that the numbers of cTfh cells were predominantly affected by abatacept treatment. Importantly, ICOS expression on $CD4^+$ T cells also declined, both in peripheral blood and parotid gland tissue. The decrease in ICOS expression on cTfh cells correlated significantly with the reduction in systemic disease activity, as measured by ESSDAI, over time.

PATIENTS AND METHODS

Study population

This was a prospective, single-center, open-label study of patients with early and active pSS (n=15) [20]. All patients fulfilled the American-European Consensus Group criteria for pSS [22]. Patients had not previously received any biologic disease-modifying antirheumatic drug, and immunosuppressants, including hydroxychloroquine and oral prednisone, were discontinued before inclusion. Baseline characteristics of the patients are displayed in Supplementary Table 1. In addition, 15 age- and sex-matched healthy individuals (mean ± SD age 45 ± 11 years; 12 women) were included as healthy controls. All patients and healthy controls provided written informed consent. The study was

approved by the Medical Ethics Committee of the University Medical Center Groningen (METc2009.371).

Study procedures

Patients were treated with abatacept for 24 weeks (~10 mg/kg by intravenous infusion on days 1, 15, 29 and every 4 weeks thereafter) [20]. Follow-up was conducted at weeks 4, 12 and 24 (while receiving treatment) and weeks 36 and 48 (after completing treatment). Serum and EDTA and lithium-heparinized blood samples were obtained at baseline and during follow-up. Peripheral blood mononuclear cells (PBMCs) were immediately isolated from lithium-heparinized blood as previously described [23], cryopreserved using Nalgene Mr. Frosty boxes, and stored at -196°C. Parotid gland biopsy specimens were obtained at baseline and at week 24 of follow-up. Systemic disease activity was measured using ESSDAI and Clinical ESSDAI (ClinESSDAI; ESSDAI without the biological domain) [24,25].

Assessment of circulating $CD4^+$ T cell and B cell subsets

Absolute numbers of circulating $CD4^+$ T cells and B cells were measured in fresh EDTA blood samples using the MultiTest TruCount method (Becton Dickinson). Fresh blood samples were stained with antibodies directed against human CD3, CD4, CCR7 and CD45RO (Supplementary Table 2), lysed, washed, and analyzed by flow cytometry for the presence of naive ($CCR7^+CD45RO^-$), central memory ($CCR7^+CD45RO^+$), effector memory ($CCR7^-CD45RO^+$), and terminally differentiated ($CCR7^-CD45RO^-$) $CD4^+$ T cells [26]. In a separate experiment, cryopreserved PBMCs were thawed, 2 million cells were fluorescence labeled for effector memory $CD4^+$ T cell subset analysis, and 2 million cells were fluorescence labeled for B cell subset analysis (see Supplementary Table 2 for a list of the antibodies used). $CD4^+$ effector memory T cell subsets were identified by chemokine receptor patterns (Supplementary Figure 1). One patient was excluded from $CD4^+$ effector memory T cell analysis because of consistently aberrant CD45RA expression, which precluded analysis of memory subsets. Flow cytometric measurements were performed on a FACS LSRII flow cytometer (Becton Dickinson), and data were analyzed using Kaluza (Beckman Coulter) and FlowJo (Tree Star) software.

Quantification of cytokines and anti-SSA/SSB IgG titers in serum

Serum CXCL13 levels were quantified as part of a custom-made ProcartaPlex Multiplex Immunoassay (eBioscience), which also included interferon-γ (IFNγ), IL-4, IL-17, IL-22 and IL-10. Serum IL-21 levels were measured by an in-house enzyme-linked immunosorbent assay (ELISA) using purified anti-human IL-21 and biotin-conjugated anti-human IL-21

(eBioscience). Serum titers of anti-SSA and anti-SSB IgG were measured by an in-house ELISA using Ro52, Ro60 and La antigens (Euro-Diagnostica) and horseradish peroxidase (HRP)-conjugated goat anti-human IgG-Fc (Bethyl Laboratories).

Immunohistochemistry

For immunohistochemical staining of ICOS expression in salivary glands, formalin-fixed, paraffin-embedded sections (3 µm) of parotid gland tissue were incubated for 1 hour at room temperature with primary antibodies to ICOS (clone SP98; Thermo Scientific). Prior to incubation, heat-induced epitope retrieval (HIER) was performed using EDTA buffer (pH 8). Anti-ICOS antibodies were visualized using an HRP-linked detection system with diaminobenzidine (Dako), and sections were analyzed for staining using the Positive Pixel Count algorithm (version 9.1) in Aperio ImageScope (Aperio Technologies). Only foci were depicted for analysis, and the relative area stained within these foci was calculated by dividing the positive pixel count by the total pixel count. HIER was performed on consecutive sections using citrate buffer (pH 6), and sections were incubated overnight at 4°C with primary antibody to IL-21 (polyclonal; Novus biologicals) and the next day for 1 hour at room temperature with anti-CD4 (clone 1F6; Abcam). Two-color immunofluorescence visualization of staining was performed using Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 594-conjugated anti-rabbit IgG tyramide SuperBoost kits (Thermo Scientific) according to the manufacturer's protocol. Fluorescence images were acquired using a TissueFAX system and data were analyzed using TissueQuest software (TissueGnostics).

Statistical analysis

All results are presented as the median (interquartile range [IQR]). Mann-Whitney U tests were used to compare differences between patients and healthy controls. Generalized estimating equations (GEEs) with exchangeable correlation structure were used to analyze changes in laboratory parameters and associations with systemic disease activity over time within subjects [27]. Data from baseline up to week 24 were compared with baseline to assess changes while patients were receiving treatment. Data from week 24 to week 48 were compared with week 24 to assess changes when patients were no longer receiving treatment. Wilcoxon's matched pairs tests were used to compare histologic parameters at baseline and week 24. P values less than 0.05 (2-tailed) were considered significant. Statistical analysis was performed using SPSS Statistics version 23 (IBM).

RESULTS

Abatacept affects the distribution of CD4$^+$ T cell subsets

In patients with pSS, total numbers of circulating CD4$^+$ T cells at baseline were ~35% lower than in healthy controls (median 0.59 x 10^9/liter [IQR 0.40-0.85] versus 0.89 x 10^9/liter [IQR 0.82-0.96] in healthy controls; P=0.022). Systemic disease activity was relatively high in our study population, and it has previously been shown that CD4$^+$ T cell numbers are most strongly decreased in pSS patients with high ESSDAI scores [28]. Within the CD4$^+$ T cell fraction, proportions of naive and terminally differentiated CD4$^+$ T cells were not significantly altered in pSS patients at baseline compared with healthy controls (Figures 1A and C). Proportions of central memory CD4$^+$ T cells were significantly decreased (Figure 1B), whereas proportions of effector memory cells were significantly increased (Figure 1D), in pSS patients compared with healthy controls. These results indicate that the pSS patients studied had a relatively expanded effector memory CD4$^+$ T cell compartment, at the expense of central memory CD4$^+$ T cells. Consistent with this finding, it has been shown that pSS patients have a higher proportion of HLA-DR$^+$CD4$^+$ T cells, indicating recent activation of T cells [28].

Abatacept treatment resulted in a slight increase in total numbers of CD4$^+$ T cells (~5%) at week 4 only (data not shown). Proportions of effector memory CD4$^+$ T cells, and to a lesser extent central memory CD4$^+$ T cells, were significantly decreased in patients treated with abatacept (Figures 1B and D), while proportions of naive and terminally differentiated CD4$^+$ T cells were significantly increased (Figures 1A and C). The distribution of these 4 differentiation subsets was, however, not entirely normalized to the distribution seen in healthy controls (Figure 1). Also, absolute numbers of effector and central memory CD4$^+$ T cells were significantly reduced by abatacept (P=0.009 and P<0.001, respectively) (data not shown). These findings indicate that the two memory CD4$^+$ T cell subsets in particular are affected by abatacept, illustrating the dependency of memory T cell formation or maintenance on CD28-mediated costimulation [29].

Abatacept specifically reduces cTfh and Treg cell numbers in peripheral blood

Next, the various effector subsets and peripheral Treg cells that are comprised within the peripheral CD4$^+$ T cell fraction were studied in more detail. Baseline proportions of cTfh cells (CD45RA$^-$CXCR5$^+$PD-1$^+$), Th17 cells (CD45RA$^-$CXCR5$^-$CXCR3$^-$CCR4$^+$CCR6$^+$) and peripheral Treg cells (CD45RA$^-$FoxP3$^+$) were all significantly increased in pSS patients compared with healthy controls (Figure 2A). These results are consistent with our previous findings in a different study population [30]. The recently described Tph cells (CXCR5$^-$PD-1^{high}), which functionally resemble Tfh cells and provide B cell help [9],

were also significantly increased in the peripheral blood of pSS patients compared with healthy controls (Figure 2A). Their overall frequencies and numbers, however, remained much lower than cTfh cells.

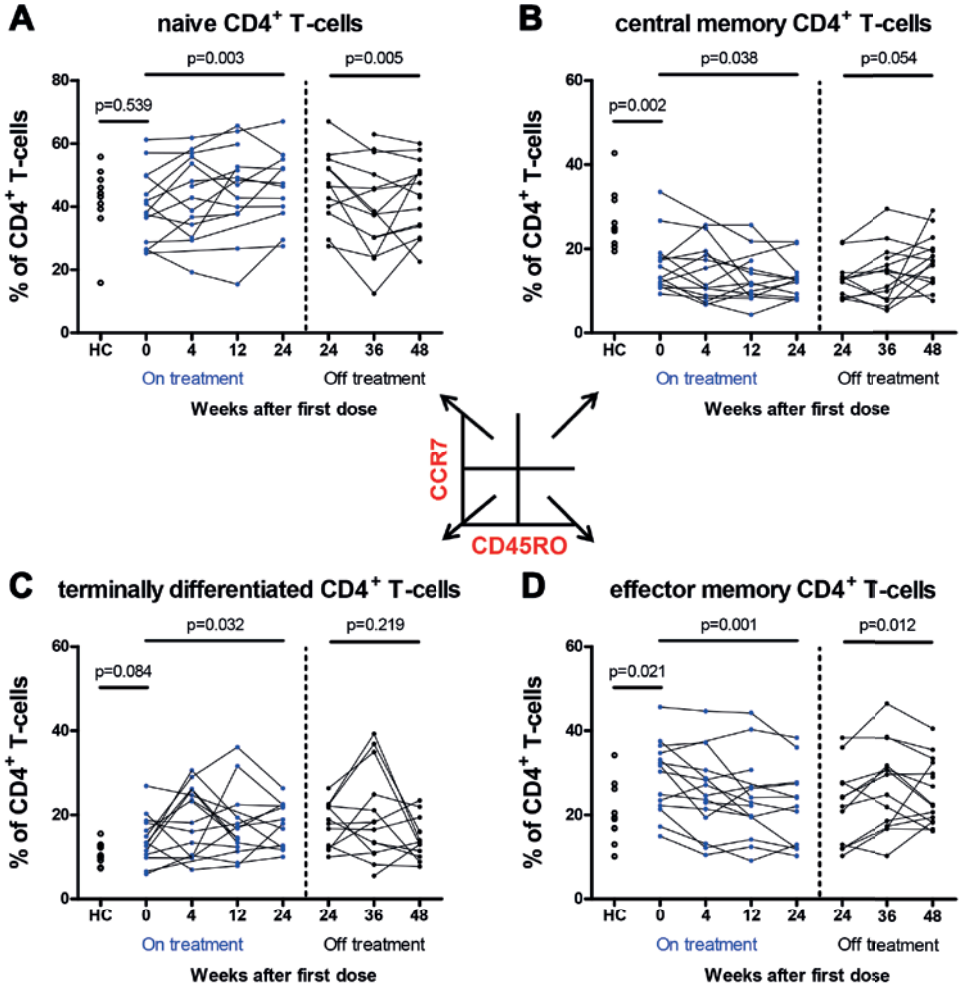

FIGURE 1 | Abatacept treatment alters the distribution of CD4⁺ T cell differentiation subsets. Proportions of naive CD4⁺ T cells (CCR7⁺CD45RO⁻) (**A**), central memory CD4⁺ T cells (CCR7⁺CD45RO⁺) (**B**), terminally differentiated CD4⁺ T cells (CCR7⁻CD45RO⁻) (**C**), and effector memory CD4⁺ T cells (CCR7⁻CD45RO⁺) (**D**) in healthy controls (HCs) and in patients with primary Sjögren's syndrome during treatment and after completing treatment are shown. Circles represent individual subjects; horizontal lines show the median. Changes in frequencies over time within patients during treatment (weeks 4-24) compared with baseline (week 0), and after treatment (weeks 36 and 48) compared with week 24, were analyzed by generalized estimating equation.

To explore the activation state of effector T cell subsets, we also analyzed the expression levels of ICOS on these cells. All effector subsets from pSS patients expressed significantly higher levels of ICOS than did healthy controls (data not shown), with the highest relative increase in expression seen on cTfh cells (Figure 2B). Apparently, effector $CD4^+$ T cells from pSS patients are in a hyperactive state, which enhances their capacity to interact with antigen-presenting cells. ICOS expression was also present on peripheral Treg cells, but no significant difference in expression level between pSS patients and healthy controls was observed.

To assess the extent to which abatacept modulates T cell activation in pSS, we examined different $CD4^+$ effector memory subsets at various time points while patients were receiving treatment and after they had stopped treatment. The most pronounced effects of abatacept were seen on cTfh cells. Treatment resulted in a significant decrease in absolute numbers and proportions of this subset over time (Figures 2D and E). Absolute numbers and proportions of peripheral Treg cells also declined in patients treated with abatacept ($P<0.001$) (see Supplementary Figure 1), while absolute numbers and proportions of Th1, Th2, and Th17 cells were not significantly affected (Supplementary Figure 1). The decrease in cTfh cells during treatment was accompanied by normalization of ICOS expression levels on these cells to levels seen in healthy controls (Figures 2F and G). The decrease in ICOS levels was further reflected by a decrease in the proportion of $CXCR5^+PD\text{-}1^+ICOS^+CD4^+$ cells (Figure 2H). ICOS expression on other effector subsets and on peripheral Treg cells was also reduced (data not shown).

Although the proportions of Tph cells were also decreased in patients treated with abatacept ($P=0.003$ for week 0-24, by GEE), their absolute numbers only tended to decrease ($P=0.074$) (Figure 3B). Thus, Tph cells were less affected by abatacept than ($PD\text{-}1^{high}$) cTfh cells (Figure 3C). This selective decrease in cTfh cells was reflected by an increased ratio of $CXCR5^-PD\text{-}1^{high}$ Tph cells to $CXCR5^+PD\text{-}1^{high}$ Tfh cells during treatment (Figure 3D). After patients stopped treatment, the numbers and proportions of cTfh cells, peripheral Treg cells, and Tph cells, as well as ICOS expression levels on all subsets, reverted to baseline levels, underlining their (partial) dependency on CD28 signaling.

Since cTfh cells were reduced by abatacept, and given the notion that Tfh cells support the formation of plasmablasts, this B cell subset might also be affected by treatment. Therefore, we analyzed the number of circulating $CD19^+$ B cells and the proportion of plasmablasts ($CD19^+CD27^{++}CD38^{++}$) over time. Whereas the total number of B cells remained unchanged, the proportion of circulating plasmablasts decreased significantly during treatment ($P<0.001$) (Figure 4F). A small decrease in IgD^-IgM^+ non-switched memory cells was also observed, while naive, IgD^+ non-switched memory and switched memory B cells were not significantly affected (Supplementary Figure 2).

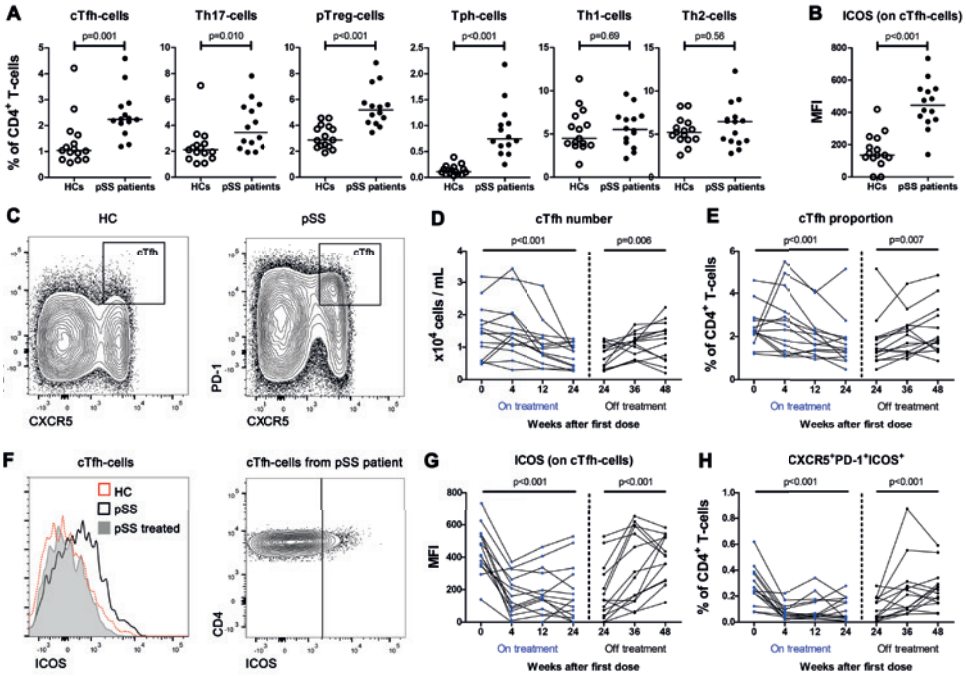

FIGURE 2 | Reduced number, proportion, and activation of circulating follicular helper T (cTfh) cells in patients with primary Sjögren's syndrome. A, Proportions of $CD4^+$ T cell subsets in healthy controls (HCs) and patients with pSS at baseline. $CD4^+CD45RA^-$ T cells were subdivided into circulating Tfh (cTfh) cells ($CXCR5^+PD\text{-}1^+$), Th17 cells ($CXCR5^-CXCR3^-CCR4^+CCR6^+$), peripheral Treg (pTreg) cells ($FoxP3^+$), peripheral helper T (Tph) cells ($CXCR5^-PD\text{-}1^{high}$), Th1 cells ($CXCR5^-CXCR3^+CCR4^-CCR6^-$) and Th2 cells ($CXCR5^-CXCR3^-CCR4^+CCR6^-$). **B**, Median fluorescence intensity (MFI) of inducible costimulator (ICOS) on cTfh cells from healthy controls and pSS patients at baseline. **C**, Gating strategy for cTfh cells within the $CD4^+CD45RA^-$ compartment in a representative healthy control sample (left) and a representative pSS sample (right). PD-1 = programmed death 1. **D** and **E**, Number (**D**) and proportion (**E**) of cTfh cells in patients with pSS during treatment and after completing treatment. **F**, Expression of the activation marker ICOS on cTfh cells from a healthy control and from a pSS patient before and after treatment (left) and gating strategy for $CXCR5^+PD\text{-}1^+ICOS^+$ cells (right). **G**, MFI of ICOS expression on cTfh cells in pSS patients during treatment and after completing treatment. **H**, Proportions of $CXCR5^+PD\text{-}1^+ICOS^+$ cells in pSS patients during treatment and after completing treatment. In **A**, **B**, **D**, **E**, **G**, and **H**, circles represent individual subjects; horizontal lines show the median. Changes over time within patients during treatment (weeks 4-24) compared with baseline (week 0), and after treatment (weeks 36 and 48) compared with week 24, were analyzed by generalized estimating equation.

Reduced serum IL-21 and CXCL13 levels in pSS patients treated with abatacept

We subsequently analyzed serum levels of signature cytokines of the various effector CD4+ T cell subsets. IL-21, the signature cytokine of Tfh cells, was detectable in 12 patients at baseline (80%). In these patients, abatacept treatment significantly decreased IL-21 levels (Figure 4A). In addition to IL-21, Tfh cells can produce the homeostatic chemokine CXCL13. Serum levels of this chemokine were also significantly reduced by abatacept

treatment (Figure 4B). After patients stopped treatment, serum levels of both IL-21 and CXCL13 returned to baseline values (Figures 4A and B). Signature cytokines of other $CD4^+$ effector subsets were not significantly altered by treatment (IL-17 and IL-22) or remained undetectable in serum at all time points (IFNγ, IL-4, and IL-10), which may be a consequence of the sensitivity of the assay.

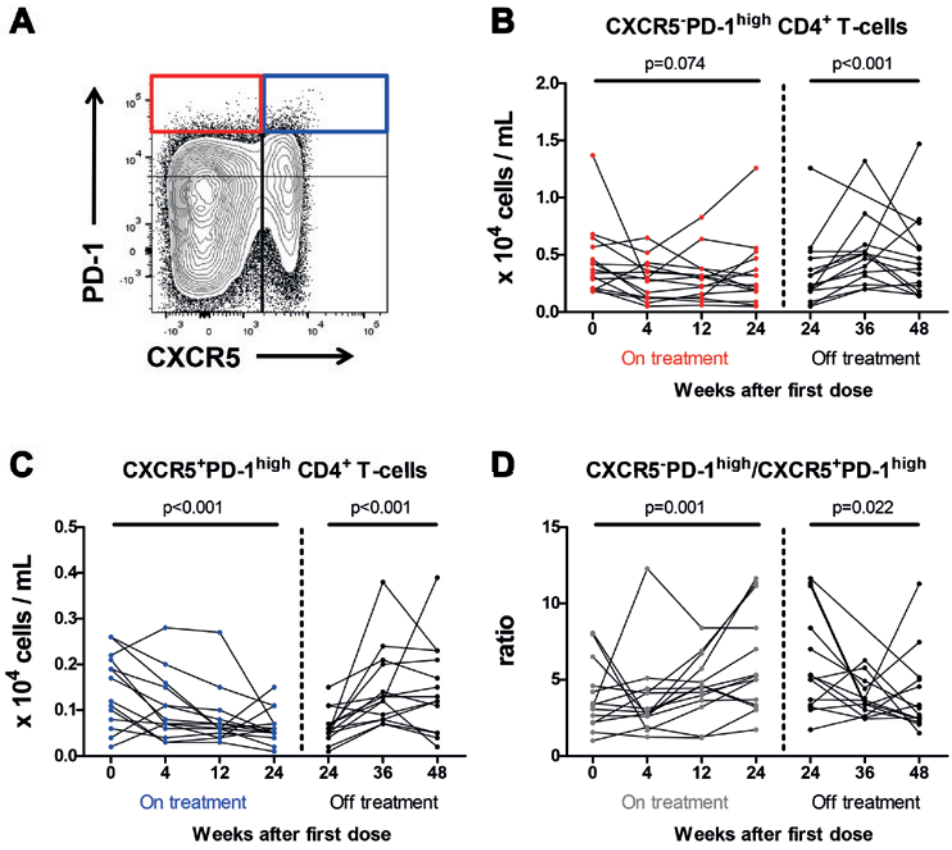

FIGURE 3 | Abatacept treatment affects numbers of peripheral helper T (Tph) cells less than numbers of PD-1^{high} circulating follicular helper T (Tfh) cells in patients with primary Sjögren's syndrome (pSS). A, Gating strategy for $CXCR5^-$PD-1^{high} cells (red) and $CXCR5^+$PD-1^{high} cells (blue) within the $CD45RA^-CD4^+$ memory compartment. **B** and **C**, Absolute numbers of Tph cells ($CXCR5^-$PD-1^{high} $CD4^+$) (**B**) and PD-1^{high} cTfh cells ($CXCR5^-$PD-$1^{high}CD4^+$) (**C**) in patients with pSS during treatment and after completing treatment. **D**, Ratio of Tph cells to cTfh cells in patients with pSS during treatment and after completing treatment. In **B-D**, circles represent individual subjects; horizontal lines show the median. Changes over time within patients during treatment (weeks 4-24) compared with baseline (week 0), and after treatment (weeks 36 and 48) compared with week 24, were analyzed by generalized estimating equation. PD-1 = programmed death 1.

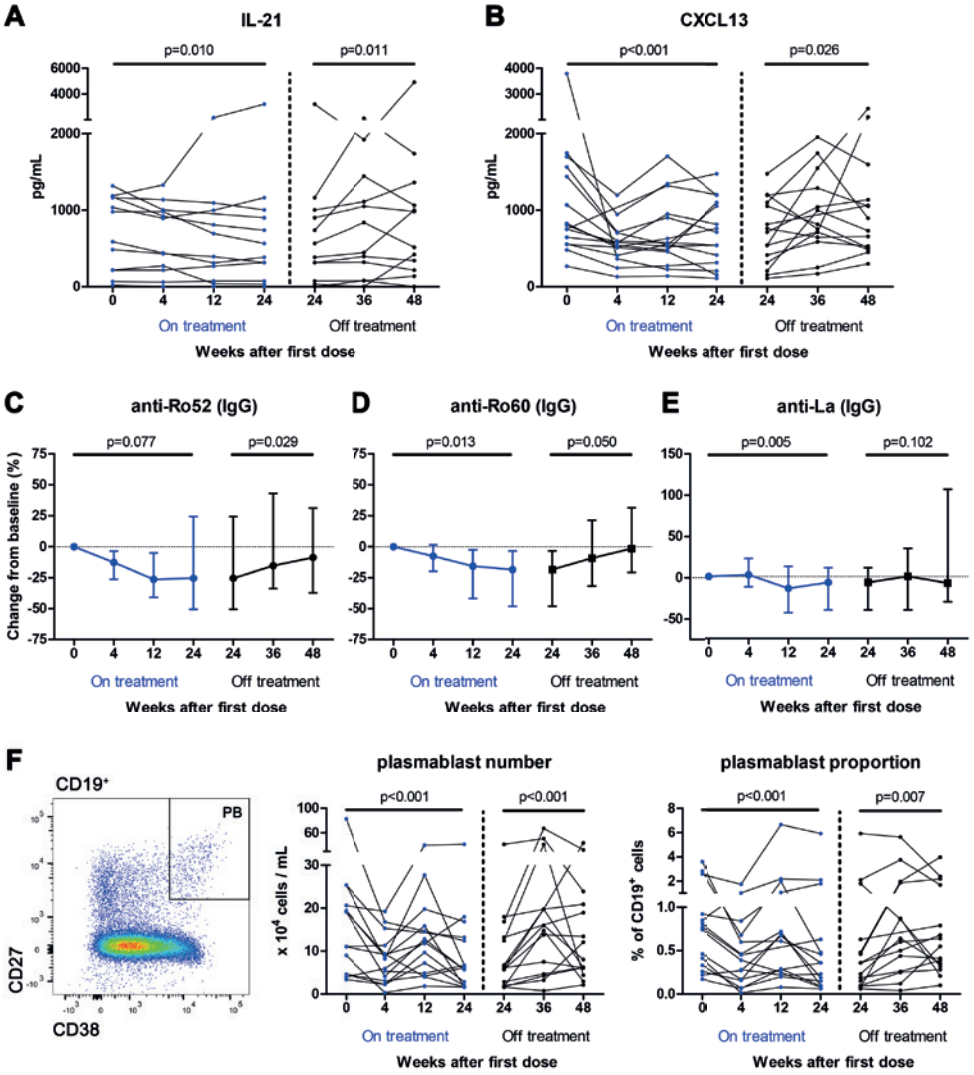

FIGURE 4 | Reduced circulating follicular helper T cell-related cytokines, autoantibodies and plasmablasts (PB) in patients with primary Sjögren's syndrome (pSS) treated with abatacept. **A**, Serum interleukin-21 (IL-21) levels, measured by an enzyme-linked immunosorbent assay (ELISA), in 12 pSS patients during treatment and after completing treatment. **B**, Serum CXCL13 levels, measured by multiplex bead assay, in 15 pSS patients during treatment and after completing treatment. In **A** and **B**, circles represent individual patients; horizontal lines show the median. **C-E**, Serum concentrations of autoantibodies directed against Ro52 (**C**), Ro60 (**D**) and La (**E**), as measured by ELISA. The percent change was calculated using the baseline concentration as the reference. Values are the median(interquartile range). **F**, Left, Identification of plasmablasts as $CD19^{+}CD27^{++}CD38^{++}$ cells. Middle and right, Number (middle) and proportion (right) of plasmablasts in pSS patients during treatment and after completing treatment. Circles represent individual patients; horizontal lines show the median. P values were determined by Mann-Whitney U test. Changes over time within patients during treatment (weeks 4-24) compared with baseline (week 0), and after treatment (weeks 36 and 48) compared with week 24, were analyzed by generalized estimating equation.

Abatacept affects SSA and SSB antibody titers

Since serum IL-21 levels, numbers of cTfh cells, and plasmablasts were all reduced by treatment, we next investigated whether concentrations of autoantibodies in serum were also affected by abatacept. Consistent with the observed decrease of ~20% in total IgG [20], levels of IgG antibodies to both isoforms of SSA (Ro52 and Ro60), and to SSB (La) were decreased to a similar extent during treatment (Figures 4C-E). Anti-Ro52 and anti-Ro60 levels, but not anti-La levels, increased significantly after cessation of treatment. Thus, abatacept limits maintenance of IgG production, including autoantibodies, which is likely a result of reduced Tfh cell-mediated activation of B cells.

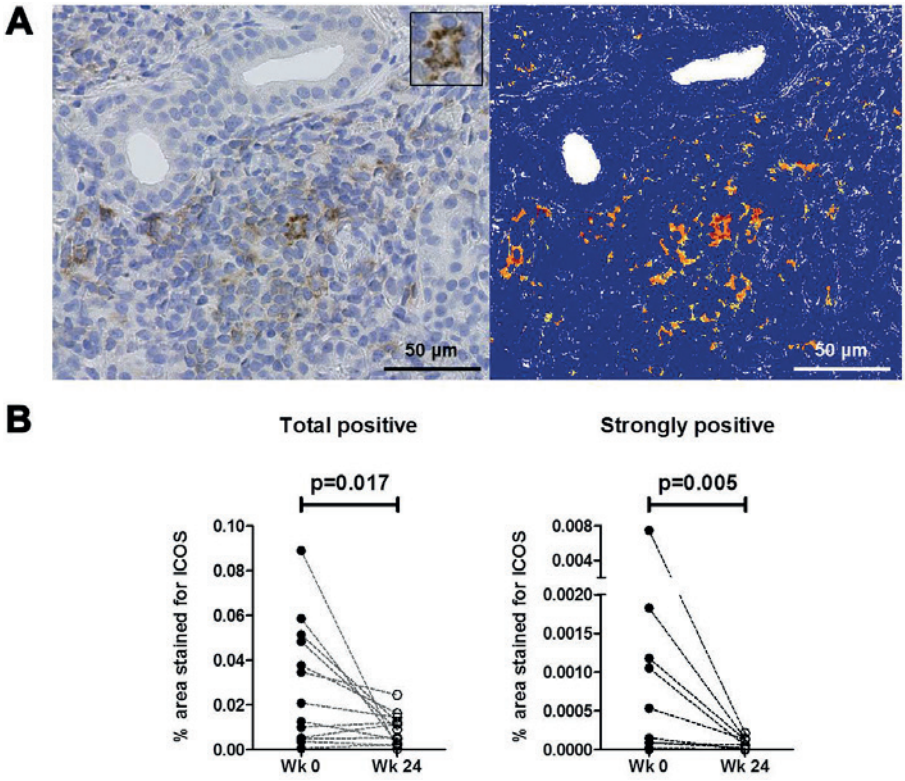

FIGURE 5 | Diminished expression of inducible costimulator (ICOS) protein in parotid gland tissue from patients with primary Sjögren's syndrome (pSS) treated with abatacept. A, Left, Representative image of ICOS staining (brown), counterstained with hematoxylin, in parotid gland tissue from a patient with pSS at baseline. Boxed area shows a cell that is strongly positive for ICOS. Right, Positive pixel count analysis using Aperio ImageScope software, which discriminates negative (blue), weak positive (yellow), positive (orange), and strong positive (red) pixels. **B**, Quantification of total positive staining and strong positive staining for ICOS within periductal foci at baseline and week 24. Differences in staining between baseline and week 24 were analyzed by Wilcoxon's matched pairs test.

Decreased ICOS expression in the parotid glands of patients treated with abatacept

To explore whether the effects on cTfh cells were also reflected in the inflamed glandular tissue, we analyzed protein expression of ICOS and IL-21 in the parotid gland tissue of patients with pSS before and after treatment. We recently showed that focus score and absolute numbers of $CD3^+$ T cells/mm^2 parenchyma were not altered by treatment [31]. In the present study, immunohistochemical staining revealed that ICOS expression within foci was significantly decreased by treatment (Figure 5). Different levels of staining (weak, medium and strong) could be distinguished at baseline; after treatment, strong staining was nearly absent (Figure 5B). In contrast to ICOS expression, numbers of $CD4^+IL\text{-}21^+$ cells in parotid glands were not significantly affected by treatment (data not shown).

Correlation of changes in laboratory parameters with systemic disease activity

ESSDAI and ClinESSDAI were used to assess the clinical relevance of the Tfh cell-related changes observed during abatacept treatment. The decline in ICOS expression levels on cTfh cells, but not numbers of cTfh cells, was significantly associated with the decrease in ESSDAI and ClinESSDAI scores (Figure 6). Changes in serum levels of CXCL13, anti-Ro60 and rheumatoid factor (RF) induced by abatacept over time were also significantly associated with a decrease in ESSDAI and ClinESSDAI, whereas changes in the number of peripheral Treg cells and levels of total IgG, IL-21, anti-Ro52, and anti-La were not (Figure 6).

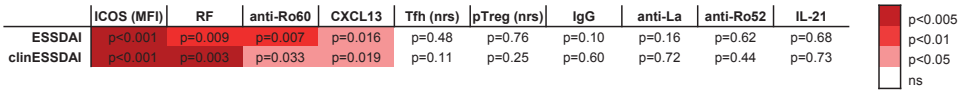

FIGURE 6 | Associations between changes in systemic disease activity and systemic laboratory parameters over time in patients with primary Sjögren's syndrome treated with abatacept. Associations between systemic disease activity, as measured by the European League Against Rheumatism (EULAR) Sjögren's syndrome Disease Activity Index (ESSDAI) and Clinical ESSDAI (ClinESSDAI; the ESSDAI without the biological domain), and systemic laboratory parameters that change over time during abatacept treatment (weeks 0-24) were analyzed by generalized estimating equation. The dependent variables (ESSDAI and ClinESSDAI) were square root transformed before they were entered into the equation. P values less than 0.005 were considered significant after Bonferroni correction. Rheumatoid factor (RF) levels were measured in kIU/liter. ICOS = inducible costimulator; MFI = median fluorescence intensity; Tfh = follicular helper T cell; nrs = number of cells; pTreg = peripheral Treg cells; IL-21 = interleukin-21; NS = not significant.

DISCUSSION

Previously, we showed in an open-label study that abatacept is effective for the treatment of pSS [20]. In the present study, we demonstrate that abatacept predominantly targets cTfh cells, which may help to explain the underlying mechanism of clinical efficacy. Abatacept resulted not only in a decrease in the numbers and activation state of cTfh cells, but also in reduced serum levels of Tfh cell-related cytokines (i.e., IL-21 and CXCL13). A decrease in cTfh cells likely contributed to the observed decrease in circulating plasmablasts and anti-SSA/SSB titers. Furthermore, abatacept reduced the numbers of peripheral Treg cells and tended to reduce the numbers of the novel Tph cell subset, defined as $CXCR5^{-}PD\text{-}1^{high}$, but did not affect Th1, Th2, or Th17 cells. Taken together, these findings indicate that the effects of abatacept are selective and reduce Tfh cell activity, thereby attenuating Tfh cell-dependent B cell hyperactivity in pSS patients.

Although the number of patients studied was small (n=15), baseline proportions of cTfh cells were significantly elevated in the patients compared with healthy controls. These results are consistent with previous findings in pSS [6,14,32]. A new observation is that cTfh cells from pSS patients have a 3-fold higher expression level of ICOS compared to healthy controls. Since ICOS is highly up-regulated upon activation [11,33], together with CXCR5 and PD-1, Tfh cells in patients with pSS are likely in a hyperactive state. Increased ICOS expression by $CXCR5^{+}CD4^{+}$ T cells provides a stronger costimulatory signal to the B cell via ligation of ICOSL [34,35]. In turn, this interaction promotes expression of IL-21 by Tfh cells [36]. It is plausible that ICOS/ICOSL-mediated cross-talk between Tfh cells and B cells is a critical step in pSS-associated B cell hyperactivity. The importance of a positive-feedback loop between B cells and T cells in autoimmune pathology is illustrated by findings that BTK overexpression in B cells leads to systemic autoimmunity in mice, together with higher numbers of splenic Tfh cells and higher expression levels of ICOS on T cells, compared with wild-type mice [37].

Importantly, we demonstrate a selective decrease in the numbers and proportions of cTfh cells in pSS patients treated with abatacept, to levels normally seen in healthy controls. Furthermore, we showed that activation of cTfh cells, as revealed by ICOS expression, was strongly diminished by this treatment. In addition to its direct role in B cell activation, ICOS expression is essential for the maintenance and accumulation of Tfh cells [38,39]. Normalization of ICOS levels in pSS patients by abatacept, as a result of less CD28-mediated activation, may therefore be the key event leading to fewer Tfh cells. In RA patients, a decrease in cTfh cells has also been observed after abatacept treatment [40]. In the latter study, only one time point after treatment was evaluated, and expression of ICOS and other possible activation markers were not measured.

From animal studies it has become clear that abatacept limits activation of $CD4^{+}$ T cells after antigen exposure [19,41]. More specifically, it also prevents antigen-induced

expression of ICOS and PD-1 on $CD4^+$ T cells, and autoantibody formation in mice [41]. The latter observations are consistent with the decrease in total IgG and RF observed in our cohort of abatacept-treated pSS patients [20] as well as with the decline in ICOS expression on $CD4^+$ T cells, plasmablasts and anti-SSA and anti-SSB titers observed in the present study. Abatacept-treated patients with RA in remission also have reduced serum titers of autoantibodies [42]. It has been proposed that abatacept blocks survival signals to CD28-expressing autoreactive plasma cells, leading to apoptosis and consequently also to a decline in autoantibody titers [43,44]. However, we were unable to detect $CD28^+$ plasma cells in parotid gland tissue from pSS patients (Kroese FGM: unpublished observations). The other explanation is that at least some of the autoantibody-producing plasma cells are relatively short-lived cells, which are not replaced by newly generated plasma cells in the absence of T cell help. In support of this notion, anti-Ro60 clonotypes in the serum of pSS patients are continuously replaced, which suggests that at least part of these IgG autoantibodies are derived from short-lived plasma cells [45].

In addition to the effects on cTfh cells, abatacept reduces numbers and proportions of peripheral Treg cells. This can be explained by the requirement of CD28 signaling for peripheral Treg survival and proliferation [46]. Consistent with our findings, Adler et al [21] observed a decrease in $CD3^+FoxP3^+$ cell numbers in labial salivary gland sections from pSS patients after abatacept treatment. It is likely that the peripheral and local decrease in peripheral Treg cells is an effect of less $CD4^+$ T cell activation. Since the suppressive capacity of the remaining Tregs cells is not known, the functional implication of this effect is not clear.

Low numbers of Tfh cells ($CD3^+Bcl6^+$) have also been detected in minor salivary glands of pSS patients [47]. Here they occur in close association with $Bcl6^+$ GC B cells. In our study population, $Bcl6^+$ cells were rarely detected in parotid gland tissue sections in the absence of GCs (data not shown). Since ICOS is highly expressed on Tfh cells and IL-21 is the signature cytokine of Tfh cells, we alternatively analyzed the presence of $ICOS^+$ cells and $CD4^+IL\text{-}21^+$ cells by immunohistochemistry/immunofluorescence in parotid gland tissue before and after treatment with abatacept. ICOS staining was significantly lowered by treatment, and strongly ICOS-positive cells, which may represent Tfh cells, were nearly absent after treatment. As mentioned above, these reduced levels may result in less efficient costimulation of antigen-presenting cells in salivary gland tissue. Tfh cells in follicles require continuous signals from B cells via ICOS/ICOSL ligation. When ICOSL is blocked, Tfh cells lose CXCR5 expression and migrate back to the T cell zone, which results in a collapse of GCs [38]. Interestingly, in our study population a decrease in GCs in parotid gland tissue was seen after abatacept treatment [31].

Although ICOS expression in parotid gland tissue is reduced by treatment, the number of $CD4^+IL\text{-}21^+$ cells does not change significantly. Apparently, $CD4^+$ T cells that reside in the gland are still activated by antigen-presenting cells and stimulated

to produce cytokines via costimulatory pathways other than CD28-CD80/86 and ICOS/ICOSL, or IL-21-production is maintained by autocrine regulation and/or continuous presence of IL-6. Possibly, IL-21 is still produced after abatacept treatment by other cells than classic ICOS^{high} Tfh cells, since IL-21 production is not restricted to Tfh cells [16]. For example, IL-21-producing CXCR5^{-}PD-1^{high} CD4^{+} T cells were abundant in joint tissue of RA patients, and their frequency was higher than PD-1^{high}CXCR5^{+} CD4^{+} Tfh cells [9]. In addition, a novel population of *Il21*+ Th1 cells was recently identified in salivary gland tissue from NOD mice [48], a murine model of SS, and this population may also be responsible for glandular IL-21 production in pSS patients.

In the present study, we showed that the decline in ICOS expression levels on cTfh cells was significantly associated with the decrease in systemic disease activity. Changes in the levels of CXCL13 and autoantibodies (RF and anti-Ro60) were also associated with a decrease in systemic disease activity. It is known that positivity for RF and anti-SSA, of which anti-Ro60 usually makes up the largest part, are both associated with the presence of extraglandular symptoms [49]. Also, serum levels of CXCL13 have been associated with systemic disease activity in pSS [50]. Attenuation of the hyperactivated state of Tfh cells by abatacept, with a concomitant decrease in serum RF, anti-SSA, and CXCL13 levels, probably underlies the amelioration of systemic disease activity. How these factors influence systemic disease activity is not yet understood and remains to be elucidated.

In contrast, the presence of glandular IL-21-producing CD4^{+} T cells was not affected by treatment in this study. Perhaps patients should be treated for a longer period of time to significantly reduce the influx of newly formed effector T cells into the glands. Alternatively, simultaneous blockade of other costimulatory pathways or direct targeting of IL-6 or IL-21 could be of additional value to target Tfh cell activity in the glands. In conclusion, abatacept affects cTfh cell activation and ICOS expression and concomitantly results in amelioration of disease activity. Our findings support the notion that Tfh cell-dependent B-cell hyperactivity plays a central role in pSS pathogenesis. We postulate that the cross-talk between Tfh and B cells is a key target for successful therapeutic interventions in pSS.

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SUPPLEMENTARY MATERIALS

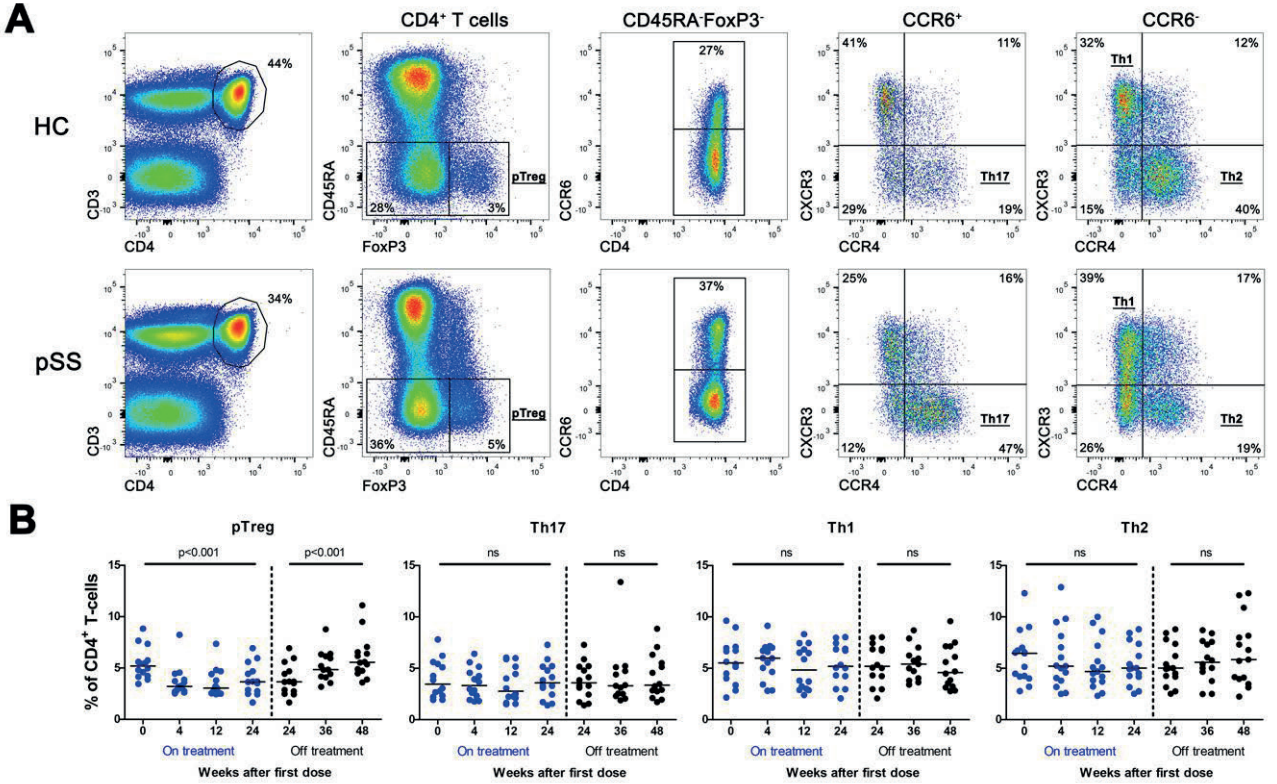
SUPPLEMENTARY TABLE 1 | Baseline characteristics of pSS patients.

Characteristic	pSS patients (*n*=15)
Age, median (IQR), years	43 (32-51)
Female gender, n (%)	12 (80)
Disease duration (months), median (IQR)	11 (7-36)
ESSPRI, median (IQR)	7.5 (6-8)
ESSDAI, median (IQR)	11 (8-14)
clinESSDAI, median (IQR)	11 (9-17)
IgG (g/L), median (IQR)	20.2 (15.3-26.7)
Anti-Ro/SSA positive, n (%)	15 (100)
Anti-La/SSB positive, n (%)	12 (80)
RF (kIU/L), median (IQR)	43 (20-184)
SWS (mL/minute), median (IQR)	0.31 (0.02-1.47)
Focus score, median (IQR)	3.1 (1.5-5.0)

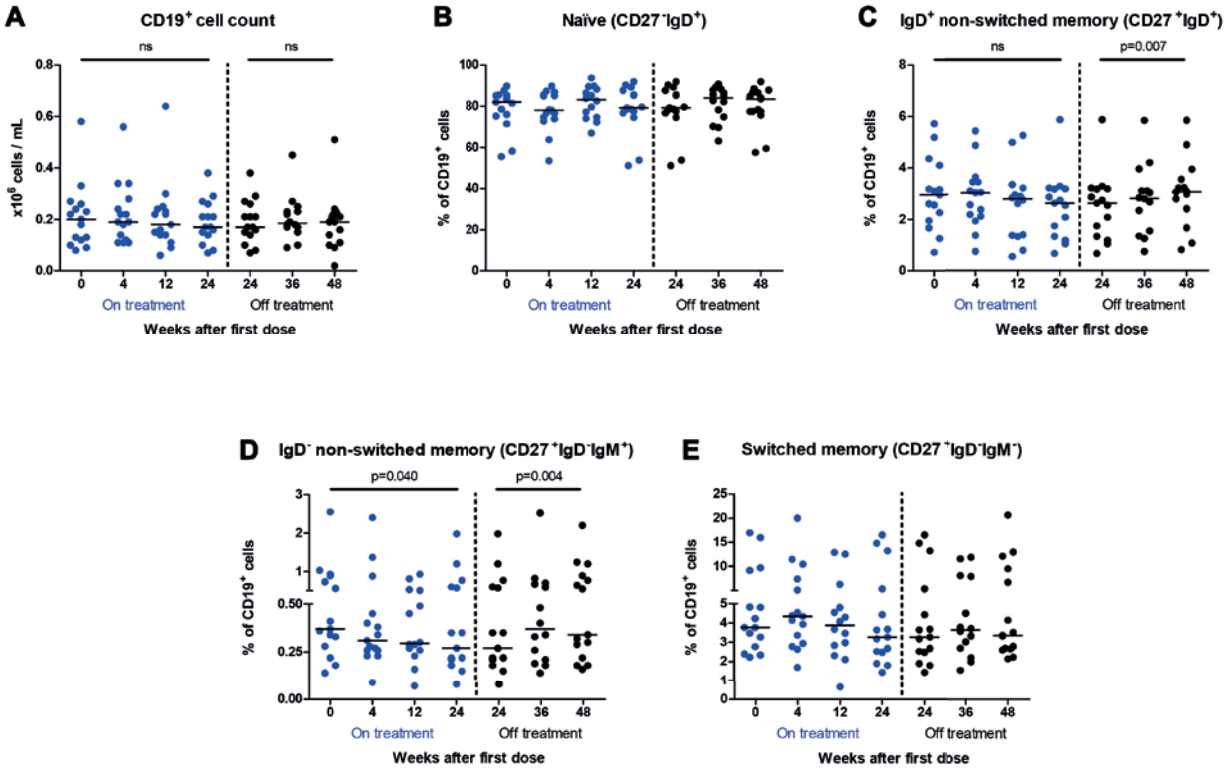
ESSPRI, European League Against Rheumatism (EULAR) Sjögren's syndrome Patient Reported Index; ESSDAI, European League Against Rheumatism (EULAR) Sjögren's syndrome Disease Activity Index; RF, rheumatoid factor; SWS, stimulated whole saliva. Focus score in parotid gland tissue.

SUPPLEMENTARY TABLE 2 | Antibodies used for flow cytometry.

Antigen	Fluorochrome	Clone	Company
$CD4^+$ T-cell differentiation subset analysis			
CD3	APC-eF780	UCHT1	eBioscience
CD4	eF605	OKT4	eBioscience
CCR7	FITC	3D12	eBioscience
CD45RO	AF700	UCHL1	Biolegend
Effector memory $CD4^+$ T-cell subset analysis			
CD3	APC-eF780	UCHT1	eBioscience
CD4	AF700	OKT4	eBioscience
CD45RA	BV650	HI100	BD Biosciences
FoxP3	PE	236A/E7	eBioscience
CXCR3	BV711	1C6/CXCR3	BD Biosciences
CCR6	APC	11A9	BD Biosciences
CXCR5	PerCP-Cy5.5	RF8B2	BD Biosciences
CCR4	FITC	205410	R&D Systems
PD-1	BV786	EH12.1	BD Biosciences
ICOS	PE-Cy7	ISA-3	eBioscience
Fixable viability dye	eF506	-	eBioscience
B-cell subset analysis			
CD19	PerCP-Cy5.5	SJ25C1	BD Biosciences
CD27	BV421	MT272	BD Biosciences
CD38	APC	HIT2	BD Biosciences
IgD	APC-H7	IA6-2	BD Biosciences
IgM	BV605	G20-127	BD Biosciences



SUPPLEMENTARY FIGURE 1 | Effect of abatacept treatment on circulating pTreg-cells, Th17-cells, Th1-cells and Th2-cells. (**A**) Gating strategy for peripheral Treg (pTreg)-cells, Th17-cells, Th1-cells and Th2-cells in a representative healthy control and patient sample. First, pTreg-cells ($CD3^+CD4^+CD45RA^-FoxP3^+$) and $FoxP3^-$ memory $CD4^+$ T-cells were gated ($CD3^+CD4^+CD45RA^-FoxP3^-$). Th17-, Th1- andTh2-cells were distinguished based on the expression pattern of surface chemokine receptors. Fluorescence minus one (FMO) controls were used to set the gates. (**B**) Dot plots of the proportion of pTreg-cells, Th17-cells, Th1-cells and Th2-cells within $CD4^+$ T-cells over time are displayed. Horizontal lines indicate the median. Changes over time within patients were analyzed by generalized estimating equations on treatment (week 0-24) and off treatment (week 24-48), compared with baseline and week 24, respectively. P-value < 0.05 was considered statistically significant.



SUPPLEMENTARY FIGURE 2 | Frequencies of circulating B-cell subsets in pSS patients at baseline, on treatment and off treatment. (**A**) Absolute numbers of circulating B-cells, (**B**) frequencies of naïve B-cells ($CD27^-IgD^+$), (**C**) frequencies of IgD^+IgM^- non-switched memory B-cells ($CD27^+IgD^+$), (**D**) frequencies of IgD^+IgM^+ non-switched memory B-cells ($CD27^+IgD^+IgM^+$) and (**E**) frequencies of switched memory B-cells ($CD27^+IgD^-IgM^-$) are displayed. Horizontal lines indicate the median. If the data followed a linear course, changes over time within patients were analyzed by generalized estimating equations on treatment (week 0-24) and off treatment (week 24-48), compared with baseline and week 24, respectively. P-value < 0.05 was considered statistically significant.

9

ENHANCED BRUTON'S TYROSINE KINASE ACTIVITY IN PERIPHERAL BLOOD B LYMPHOCYTES FROM PATIENTS WITH AUTOIMMUNE DISEASE

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ABSTRACT

Objective

Bruton's tyrosine kinase (BTK) transmits crucial survival signals from the B cell receptor (BCR) in B cells. Pharmacologic BTK inhibition effectively diminishes disease symptoms in mouse models of autoimmunity; conversely, transgenic BTK overexpression induces systemic autoimmunity in mice. We undertook this study to investigate BTK expression and activity in human B cells in the context of autoimmune disease.

Methods

Using intracellular flow cytometry, we quantified BTK expression and phosphorylation in subsets of peripheral blood B cells from 30 patients with rheumatoid arthritis (RA), 26 patients with primary Sjögren's syndrome (pSS), and matched healthy controls.

Results

In circulating B cells, BTK protein expression levels correlated with BTK phosphorylation. BTK expression was up-regulated upon BCR stimulation in vitro and was significantly higher in CD27+ memory B cells than in CD27-IgD+ naive B cells. Importantly, BTK protein and phospho-BTK were significantly increased in B cells from anti-citrullinated protein antibodies (ACPA)-positive RA patients but not in B cells from ACPA-negative RA patients. BTK was increased both in naive B cells and in memory B cells and correlated with frequencies of circulating $CCR6^+$ Th17 cells. Likewise, BTK protein was increased in B cells from a major fraction of patients with pSS and correlated with serum rheumatoid factor levels and parotid gland T cell infiltration. Interestingly, targeting T cell activation in patients with pSS using the CTLA-4Ig fusion protein abatacept restored BTK protein expression in B cells to normal levels.

Conclusion

These data indicate that autoimmune disease in humans is characterized by enhanced BTK activity, which is linked not only to autoantibody formation but also to T cell activity.

INTRODUCTION

B lymphocytes play a crucial role in various systemic autoimmune diseases. This is evident from the characteristic autoantibody repertoire, the genetic associations identified, and the promising efficacy of B cell-targeted therapies in rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Sjögren's syndrome (SS). Autoantibodies directed against nuclear self antigens in SLE and SS or anti-citrullinated protein antibodies (ACPAs) in RA often appear in patient serum before the onset of clinical symptoms [1–3]. In primary SS (pSS) [4], the presence of circulating nuclear autoantibodies and germinal centers (GCs) in salivary glands correlates with disease severity [5–7]. Moreover, genetic studies have implicated several genes involved in activation and differentiation of B cells in the pathogenesis of pSS and SLE, including *BLK, BANK1, LYN* and *BAFF* [1,8,9]. Next to being precursors of plasmablasts and plasma cells that secrete autoantibodies, B cells can engage T cells by supporting follicular Th cell differentiation, antigen presentation, production of inflammatory cytokines, and induction of tertiary lymphoid structures [10,11]. Although effects of B cell-depleting regimens in pSS are inconclusive [12–14], the efficacy of neutralizing BAFF in SLE and the efficacy of rituximab (an anti-CD20 antibody that eliminates B cells) in RA indicate that intelligent modulation of B cell function or survival may be key to successful treatment of systemic autoimmunity [1,15].

Promising drug candidates for autoimmune diseases include newly developed inhibitors of Bruton's tyrosine kinase (BTK), which is a pivotal signaling molecule that directly links B cell receptor (BCR) signals to B cell proliferation and survival through activation of the transcription factor NF-kB [16]. The importance of BTK signaling to B cells is evident from the severe B cell deficiency in patients with X-linked agammaglobulinemia, who have mutations in the *BTK* gene [16,17]. The BTK small-molecule inhibitors ibrutinib and acalabrutinib have shown robust antitumor activity and limited adverse effects in clinical studies in patients with various B cell malignancies [18,19]. In murine models of SLE and RA, promising results have been obtained with BTK inhibition, which could prevent or ameliorate lupus nephritis or joint inflammation by correcting BCR-mediated B cell activation and autoantibody production [20–26] and also by dampening myeloid cell activation [18,24–26].

In the mouse, BTK protein expression levels in naive B cells are rapidly up-regulated upon BCR engagement or when B cells are activated by Toll-like receptor (TLR) or anti-CD40 stimulation [27,28]. Several mechanisms are involved in this positive feedback regulation, including microRNA-185 and NF-κB signaling [29,30]. Sufficient BTK expression is crucial for normal B cell development and function in mice [31,32]. On the other hand, appropriate regulation of BTK protein expression in B cells is crucial for maintaining immune tolerance, because CD19-hBTK-transgenic mice (with modest B cell-restricted human BTK overexpression under control of the CD19 promotor)

spontaneously develop SLE/pSS-like disease pathology [28]. In these mice, B cells are resistant to apoptosis, which aids their differentiation into autoantibody-producing plasma cells [28]. CD19-hBTK-transgenic mice manifest spontaneous GC formation, anti-nuclear autoantibodies, and lymphocyte infiltration in various organs, including salivary glands [28]. We recently found that BTK overexpression in B cells disrupts T cell homeostasis and promotes follicular Th cell differentiation, both in aging mice and in a collagen-induced arthritis model [33].

The finding that a modest increase in BTK expression in B cells is sufficient to induce systemic autoimmune disease in mice prompted us to examine BTK expression levels and regulation in B cell subsets in peripheral blood of healthy controls and patients with autoimmune disease. We show that human B cells also up-regulate BTK protein levels upon activation, and we provide data on aberrant expression levels and phosphorylation status of BTK in B cells from ACPA-positive RA patients. Likewise, we found that BTK protein was increased in B cells from a majority of patients with pSS.

PATIENTS AND METHODS

Patients and healthy individuals

Data on characteristics of the patients and healthy controls are summarized in Supplementary Table 1.

RA patients

Cohorts of ACPA-positive and ACPA-negative treatment-naive patients with early RA who were matched for the Disease Activity Score (DAS) in 44 joints [34], the presence of rheumatoid factor (RF), and the duration of symptoms have been described previously [35]. All patients met the American College of Rheumatology/European League Against Rheumatism (EULAR) 2010 classification criteria for RA [36]. Fifteen ACPA-positive and 15 ACPA-negative patients were included and matched with 15 healthy controls.

Patients with pSS

We included 26 patients with pSS who were naïve to treatment with biologic disease-modifying antirheumatic drugs (DMARDs) and who fulfilled the American-European Consensus Group criteria for SS [37]; we matched these patients with 26 healthy controls. Fifteen of the patients with pSS had participated in the previously reported Active Sjögren Abatacept Pilot (ASAP) study (METc2009.371) [38]. They had been treated with intravenous abatacept on days 1, 15, and 29 and every 4 weeks thereafter until week 24, a regimen that improved disease activity as previously reported [38]. Patients were not treated with DMARDs or prednisone for at least 1 month prior to or during this study.

Serum and peripheral blood mononuclear cells (PBMCs) were collected from all healthy controls and patients at baseline (untreated) and from the 15 abatacept-treated patients with pSS at 4, 12, 24, 36, and 48 weeks after the first dose. Experimental procedures were approved by the Erasmus Medical Center and University Medical Center Groningen medical ethics committees. All patients provided written informed consent.

Isolation and culture of human peripheral blood B cells

PBMCs were isolated by standard Ficoll-Paque (GE Healthcare) density gradients. Subsequent purification of naive B cells was performed using a human Naive B Cell Isolation Kit II (Miltenyi Biotec), and B cell purity (>95%) was verified using flow cytometry. B cells were cultured in the presence of 10 µg/mL $F(ab')_2$ goat anti-mouse IgM (Jackson ImmunoResearch), 2 µg/mL recombinant CD40L (R&D Systems), or 2 ng/mL lipopolysaccharide (LPS) for 3 days.

Flow cytometry procedures

Fluorescence labeling of cells and measurement of intracellular BTK levels were performed as described previously [28] (see Supplementary Table 2 for a list of the antibodies used). BTK gate settings were based on isotype controls, fluorescence minus one controls, and analysis of T cells, which lack BTK expression. For staining of phosphorylated BTK, PBMCs were left unstimulated or were stimulated for 30 seconds with $F(ab')_2$ anti-human IgM (20µg/mL; SouthernBiotech) and subsequently fixed with Cytofix and permeabilized with Phosflow Perm Buffer III (BD Biosciences). Flow cytometric measurements were performed on an LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Immunohistochemistry

Parotid gland biopsy specimens were obtained from 15 patients with pSS at baseline, and paraffin-embedded sections were stained with rabbit anti-human CD3 antibody (Ventana Medical Systems) and counterstained with hematoxylin using standard procedures. Numbers of $CD3^+$ cells/mm^2 were analyzed using HistoQuest software.

Laboratory assessments in serum

Baseline levels of serum immunoglobulin classes and RF were measured by nephelometry.

Statistics

Significance of continuous data was calculated using the nonparametric Mann-Whitney U test or one-way analysis of variance (ANOVA) or repeated-measures ANOVA with Tukey's multiple comparison test. P values less than 0.05 were considered significant. Significance of correlations was determined with a nonparametric Spearman test. To determine significance of the effect of abatacept on BTK levels in B cells within patients over time, a generalized estimating equation was performed using SPSS statistical software (IBM).

RESULTS

BTK protein expression is up-regulated in *in vitro*-activated human B cells and correlates with BTK phosphorylation

Upon BCR stimulation of human B cells, a signaling cascade is initiated whereby BTK is phosphorylated in its kinase domain at position Y^{551} [18]. To verify this, we stimulated fractions of PBMCs from healthy controls with anti-IgM *in vitro* and found induction of phosphorylated BTK in B cells by phosphoflow analysis (Figure 1B).

Parallel to the findings in murine B cells [27,28], we observed that anti-IgM stimulation *in vitro* also induced an upregulation of BTK protein expression in human B cells, as detected by intracellular flow cytometry; average mean fluorescence intensity values increased ~1.4-fold (Figures 1A and C). Other stimuli, including recombinant CD40L, LPS, and imiquimod (a TLR-7 agonist), induced a limited but consistently detectable increase in BTK protein levels (Figure 1A and data not shown). To further study the relationship between BTK protein expression and phosphorylation of BTK at Y^{551} in human B cells, we measured BTK protein and phospho-BTK in gated $CD19^+$ B cells from unstimulated fractions of PBMCs from 20 subjects. Signals for BTK protein and phospho-BTK varied considerably between individuals, but these were strongly correlated in individual subjects (Figure 1D and E). From these findings, we conclude that BTK protein expression levels are a sensitive and functional indicator of BTK activity in human B cells.

Differential expression of BTK protein in individual peripheral blood B cell subsets

In contrast to phosphoflow analysis, which is difficult to perform in conjunction with cell surface markers, the staining procedure for total BTK protein allows for quantification of BTK expression levels in individual B cell subsets. This enabled us to compare BTK expression between subsets of unstimulated peripheral blood B cells from healthy controls *ex vivo* (gating strategy is shown in Figure 2A). We observed that BTK expression

levels were significantly higher in antigen-experienced $CD27^+IgD^+$ and $CD27^+IgD^-IgM^+$ nonswitched memory B cells compared with $CD27^-IgD^+$ naive B cells and $CD27^+IgD^-IgM^-$ switched memory B cells (Figures 2B and C). These data indicate that BTK protein levels are tightly regulated during B cell differentiation and suggest that up-regulation of BTK expression in specific B cell subsets may have a physiologic function.

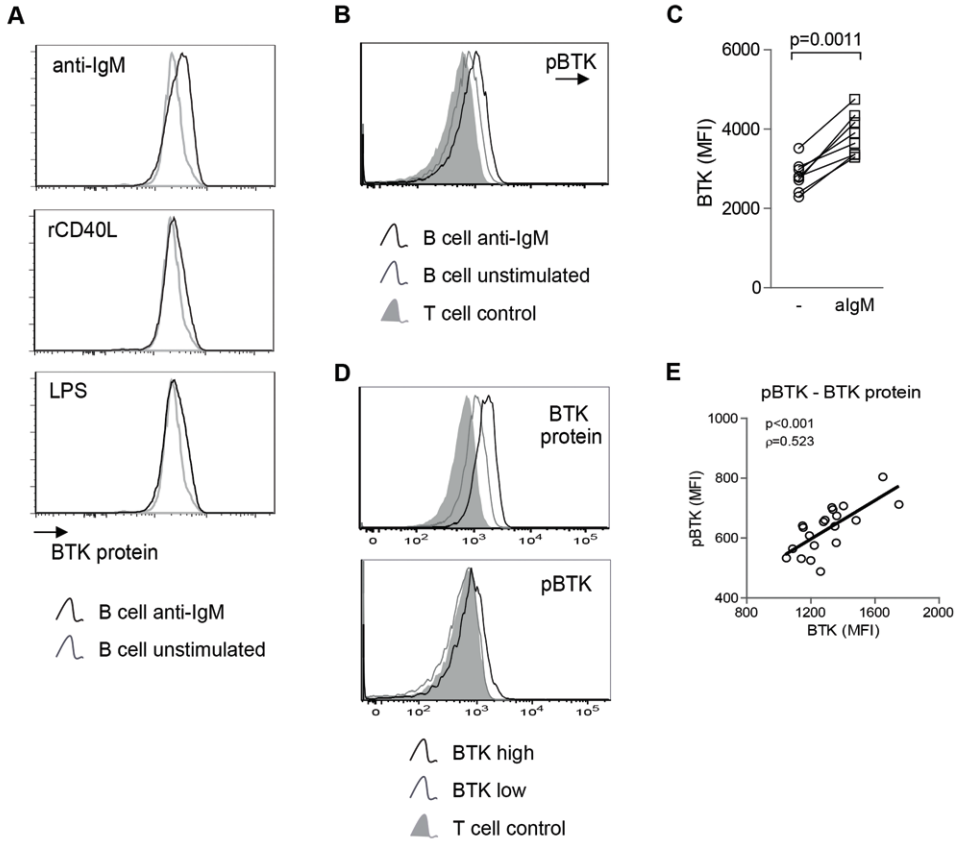

FIGURE 1 | Bruton's tyrosine kinase (BTK) protein expression and phosphorylation in human B cells are correlated and up-regulated upon B cell receptor stimulation in vitro. A, Representative graph of intracellular BTK expression in magnetic-activated cell-sorted B cells from a healthy control after 3 days of stimulation with anti-IgM (10 mg/ml), recombinant CD40L (rCD40L; 2 mg/ml), or lipopolysaccharide (LPS; 2 ng/ml). **B**, Representative graph of BTK phosphorylation in healthy control B cells that were either left unstimulated or were stimulated for 30 seconds with anti-IgM (20 mg/ml). **C**, BTK expression in magnetic-activated cell-sorted B cells from healthy controls that were either left unstimulated or were stimulated for 3 days with anti-IgM (10 mg/ml). Symbols represent specimens from individual subjects. **D**, BTK protein and phospho-BTK expression in B cells from healthy controls expressing high or low levels of BTK at baseline. **E**, Correlation between BTK protein and phospho-BTK expression in B cells from 20 subjects. Data are representative of 2 individual experiments. MFI = mean fluorescence intensity.

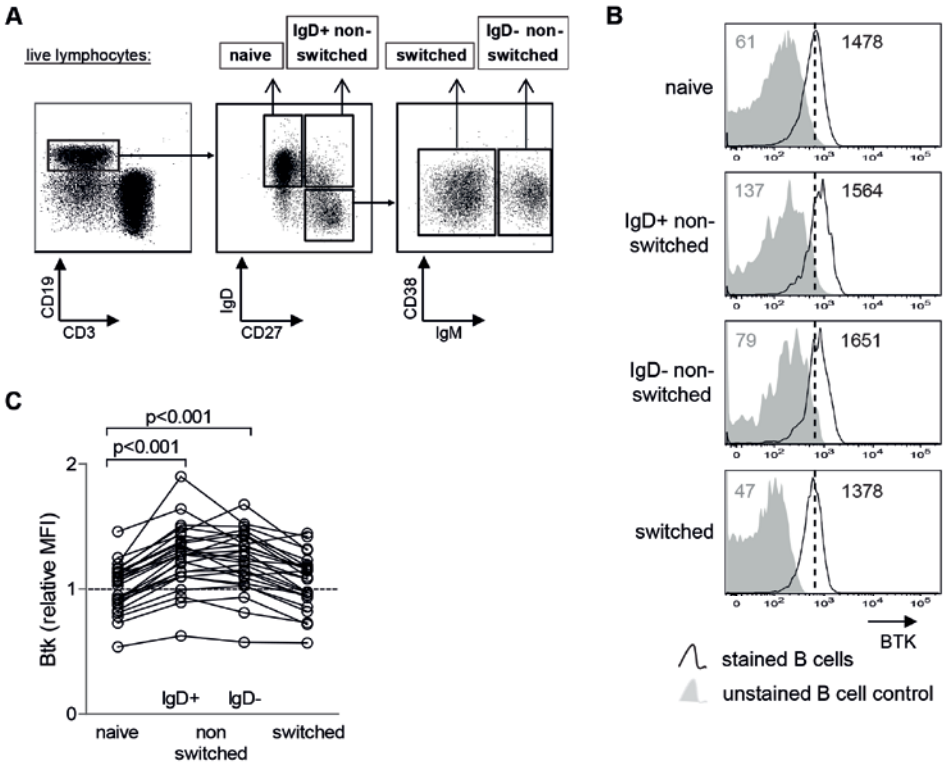

FIGURE 2 | Differential expression of Bruton's tyrosine kinase (BTK) in subsets of peripheral B cells from healthy controls. **A**, Gating strategy for naive B cells, IgD+ and IgD- nonswitched memory B cells, and switched memory B cells used throughout the study. Live lymphocytes were gated based on the absence of a cell death marker and the forward scatter and side scatter. **B**, Representative histograms of BTK expression in several B cell subsets from a healthy control. Numbers indicate mean fluorescence intensity (MFI); dashed lines represent the peak in naive B cells. **C**, BTK expression in different B cell subsets in healthy controls. Symbols represent specimens from individual subjects. Dashed line indicates average MFI in total B cells. Data are representative of 6 individual experiments. BTK protein and BTK phosphorylation are increased in peripheral blood B cells of ACPA-positive RA patients.

To investigate BTK activity in the context of autoimmune disease, we studied peripheral blood B cells from 15 ACPA-positive and 15 ACPA-negative treatment-naive RA patients who were matched for the DAS, and we included 15 age/sex-matched healthy controls (patient characteristics at baseline are displayed in Supplementary Table 1). The proportions of total B cells and the distribution over naive and various memory B cell subsets were not significantly different between RA patients and healthy controls (Figure 3A). Interestingly, when we quantified BTK expression in total B cells, we found that BTK protein levels were significantly increased (~1.4-fold) in ACPA-positive RA patients compared with ACPA-negative RA patients and healthy controls (Figure 3B) (representative flow cytometry dot plots are displayed in Supplementary Figure 1).

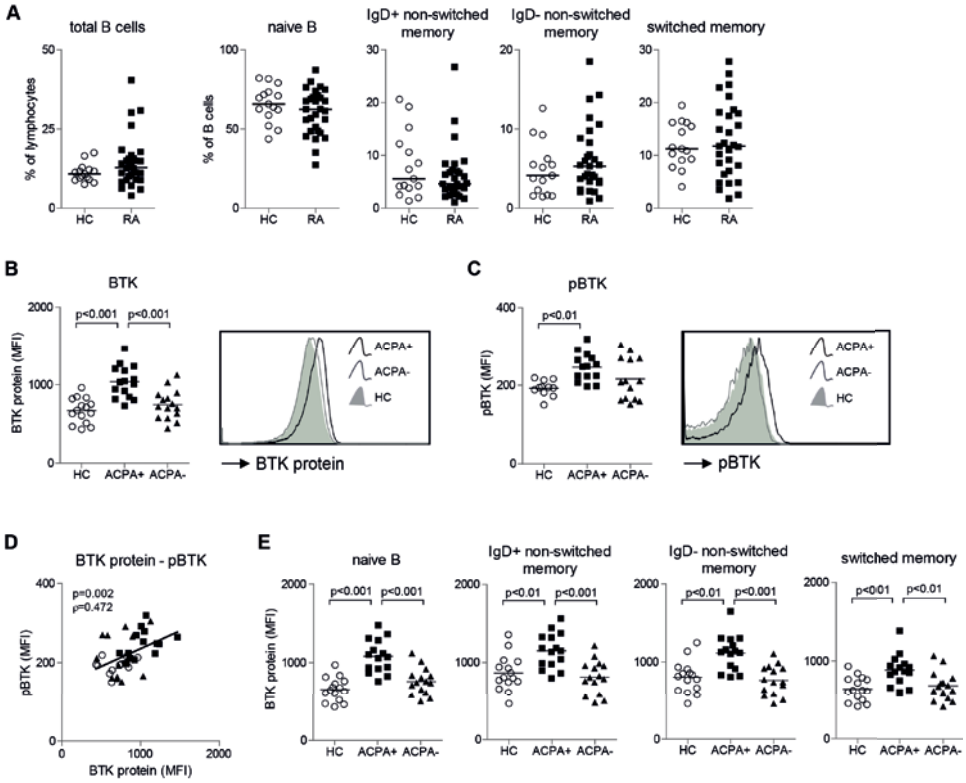

FIGURE 3 | Increased expression of Bruton's tyrosine kinase (BTK) in B cells from anti-citrullinated protein antibody (ACPA)-positive patients with rheumatoid arthritis (RA). **A**, Proportions of total B cells among live cells and proportions of naive B cells, IgD^+ and IgD^- nonswitched memory B cells, and switched memory B cells among total B cells in RA patients and healthy controls (HC). **B**, BTK protein expression in total B cells. Representative flow plots show BTK protein expression in an ACPA-positive patient, an ACPA-negative patient, and a healthy control. **C**, Phospho-BTK expression in total B cells. Representative flow plots show phospho-BTK expression in an ACPA-positive patient, an ACPA-negative patient, and a healthy control. **D**, Correlation between BTK protein and phospho-BTK expression in ACPA-positive patients (squares), ACPA-negative patients (triangles), and healthy controls (circles). **E**, BTK protein expression in the indicated B cell subsets in RA patients and healthy controls. Symbols represent specimens from individual subjects; bars show the median. MFI = mean fluorescence intensity. BTK expression levels in B cells correlate with frequencies of circulating $CCR6^+$ Th17 cells.

Similar results were found for phospho-BTK levels in unstimulated B cells, consistent with the significant correlation observed between total BTK and phospho-BTK signals (Figures 3C and D). Compared with ACPA-negative RA patients and healthy controls, ACPA-positive RA patients showed increased BTK protein expression both in $CD27^-$ IgD^+ naive B cells and in all the individual $CD27^+$ memory B cell subsets (Figure 3E). In ACPA-positive RA patients, BTK was differentially expressed between different B cell subsets, as observed in healthy controls (Supplementary Figure 2). Taken together, our intracellular flow cytometry analyses demonstrate that in ACPA-positive RA patients,

but not in ACPA-negative RA patients, relative BTK expression values were increased in all B cell subsets, including naive and memory B cells, compared with healthy controls.

Since we found that BTK-overexpressing B cells in transgenic mice have the capacity to disrupt T cell homeostasis [33], we wondered whether BTK expression levels in RA patients correlated with parameters of T cell activity. We found that BTK protein levels correlated significantly with frequencies in peripheral blood of Th17-lineage cells, which have recently been implicated in RA etiology [39] (Figures 4A and B) (gating strategy is displayed in Supplementary Figure 3). Although BTK protein levels and frequencies of follicular helper T (Tfh) cells did not correlate significantly (Figure 4C), we did observe a positive correlation of BTK protein levels with inducible costimulator (ICOS) expression on Tfh cells (Figure 4D). The latter finding is interesting, because we previously found that in mice overexpressing BTK in B cells, the expression of ICOS on T cells, including Tfh cells, was increased [33]. No correlation was found with other T cell subsets, including Th1 and Treg cells (data not shown).

In summary, the finding that BTK protein levels correlate with the frequencies of Th17-lineage cells and with Tfh cell ICOS expression suggests that BTK activity is linked to T cell activation in RA.

Peripheral blood BTK protein expression is increased in a major fraction of patients with pSS

To explore whether increased BTK activity is unique to RA or can also be found in other autoimmune disorders, we investigated BTK expression in 26 treatment-naive patients with pSS and age/sex-matched healthy controls. Because of the reported decrease in $CD27^+$ memory B cells in patients [6,40–42], we first quantified peripheral B cells subsets. Proportions of B cells in PBMCs from patients with pSS were higher than in those from healthy controls, possibly due to lymphopenia of $CD4^+$ T cells, as absolute numbers were comparable. In patients with pSS, more circulating B cells were naïve, and proportions of nonswitched and switched memory B cells were decreased (Figure 5A and data not shown).

BTK protein levels were significantly increased in $CD27^-IgD^+$ naïve B cells, $CD27^+IgD^-IgM^+$ nonswitched memory B cells, and $CD27^+IgD^-IgM^-$ switched memory B cells in patients with pSS compared with healthy controls (Figure 5B) (representative flow cytometry dot plots for total B cells are displayed in Supplementary Figure 1). In 16 of 26 patients with pSS (62%), relative BTK expression values in $CD27^-IgD^+$ naive B cells were >1.2-fold those in healthy controls. BTK levels were significantly increased in $CD86^+$ cells within this population, although proportions of $CD86^+$ naive B cells were similar to those in controls and still very low (0.25% of all naive B cells) (data not shown). BTK expression correlated with CD86 expression on naive B cells in patients with pSS and healthy controls (Supplementary Figure 4). Importantly, in patients with pSS, BTK

levels were increased even in naive CD86$^+$ B cells compared to levels in healthy controls. Patients with pSS showed differential BTK expression between different B cell subsets, similar to ACPA-positive RA patients and healthy controls (Supplementary Figure 5). In summary, in a major proportion of patients with pSS, we observed increased BTK protein expression in various B cell subsets, including naive B cells, paralleling our findings in ACPA-positive RA patients.

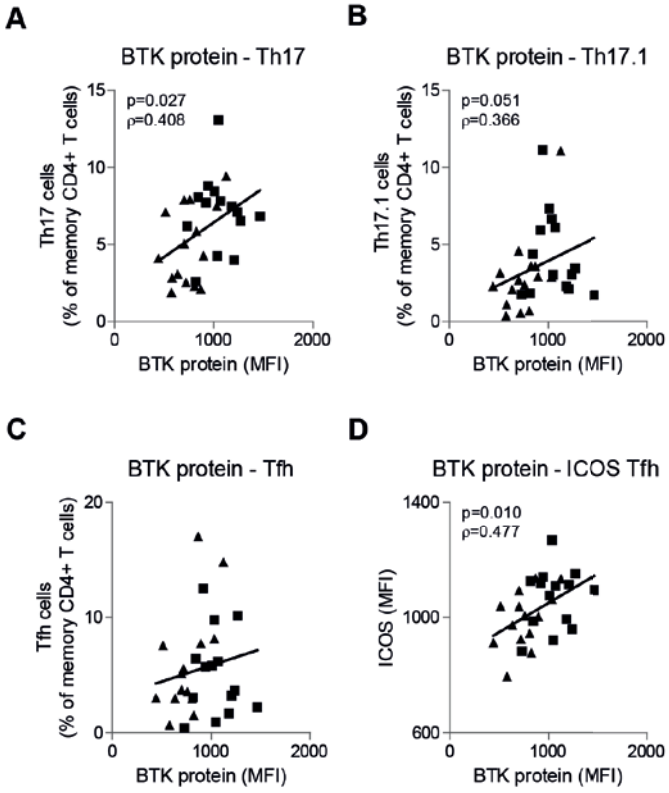

FIGURE 4 | Correlation of Bruton's tyrosine kinase (BTK) expression with Th17-lineage cells and inducible costimulator (ICOS) expression on follicular helper T (Tfh) cells. Shown are correlations between BTK protein expression and Th17 cells (CCR6$^+$CXCR3$^-$) (**A**), Th17.1 cells (CCR6$^+$CXCR3$^+$) (**B**), Tfh cells (CXCR5$^+$) (**C**), and ICOS expression by Tfh cells (**D**), measured by flow cytometry in specimens from ACPA-positive patients (squares) and ACPA-negative patients (triangles). MFI = mean fluorescence intensity.

BTK expression levels in B cells correlate with serum autoantibodies and parotid gland T cell infiltration in patients with pSS

Next, we wondered whether BTK levels correlated with clinical or immunologic parameters in patients with pSS. Scores on the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) [43] and serum IgG levels were not correlated with relative

BTK levels (data not shown). However, total serum IgM and RF levels correlated significantly with relative BTK levels in total B cells in the 26 patients with pSS (Figures 5C and D). Levels of Ro52- and Ro60-specific antibodies were higher in patients with high BTK expression, although this did not reach significance (data not shown).

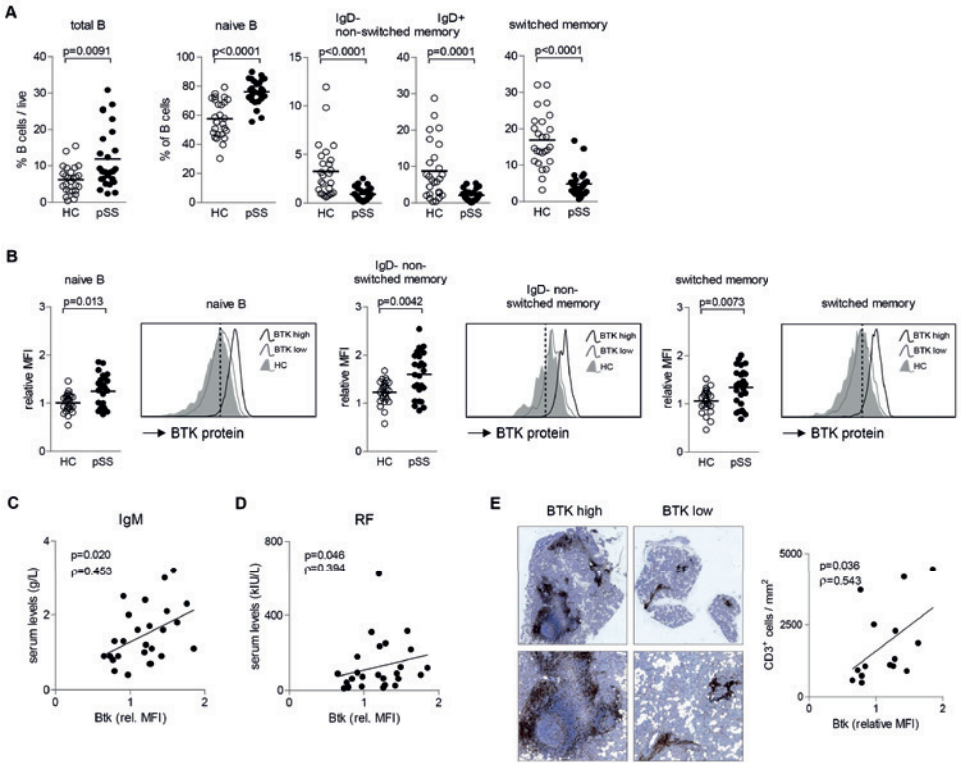

FIGURE 5 | Increased expression of Bruton's tyrosine kinase (BTK) in B cells from patients with primary Sjogren's syndrome (pSS). **A**, Proportions of total B cells among live cells and proportions of naive B cells, IgD^+ and IgD^- nonswitched memory B cells, and switched memory B cells among total B cells in patients with pSS and healthy controls (HC). **B**, BTK expression measured by intracellular flow cytometry in the indicated B cell subsets in patients with pSS and healthy controls, normalized to BTK expression in total B cells of healthy controls, which was set to 1.0. Data shown are from 6 individual experiments. Representative flow plots show BTK protein expression in B cells from 1 patient with high levels of BTK at baseline, 1 patient with low levels of BTK at baseline, and 1 healthy control. Dashed lines represent the peak of BTK expression in naive B cells from the healthy control. **C** and **D**, Correlation of relative BTK levels in B cells with total serum IgM levels (**C**) and total serum rheumatoid factor (RF) levels (**D**) in patients with pSS. **E**, Left, $CD3^+$ T cells (brown) in parotid gland biopsy specimens from 2 patients with pSS: 1 with high levels of BTK at baseline and 1 with low levels of BTK at baseline. Original magnification x 40 at top; higher-magnification views are shown at bottom. Right, Correlation of relative BTK expression in total B cells of patients with pSS with $CD3^+$ T cell infiltration of the parotid gland. Symbols represent specimens from individual subjects; bars in **A** and **B** show the median. MFI = mean fluorescence intensity.

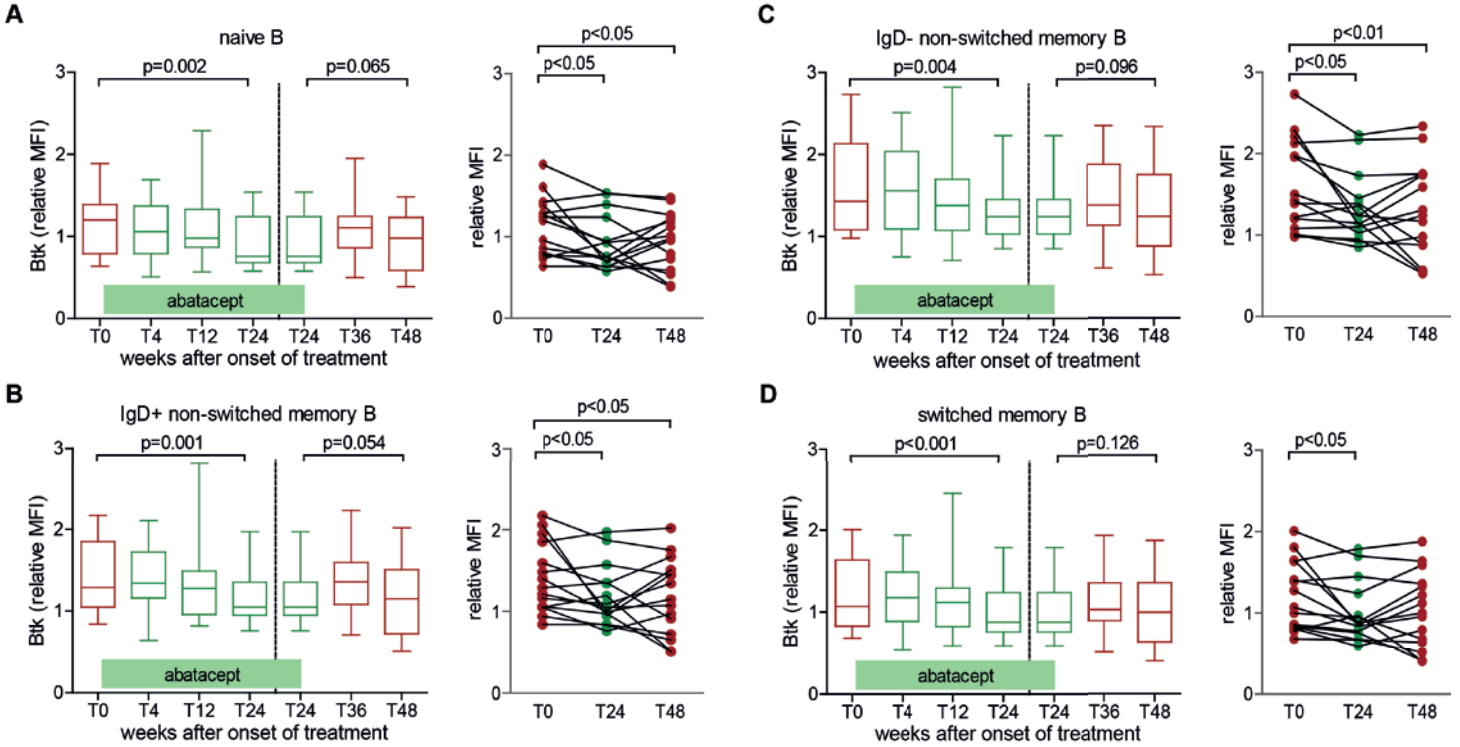

FIGURE 6 | Normalization, upon treatment with abatacept, of Bruton's tyrosine kinase (BTK) expression levels in B cells from patients with primary Sjogren's syndrome (pSS). Shown is relative BTK expression in naive B cells (**A**), IgD$^+$ nonswitched memory B cells (**B**), IgD$^-$ nonswitched memory B cells (**C**), and switched memory B cells (**D**) from 15 patients with pSS at baseline (time 0 [T0]), upon abatacept treatment (T4, T12, and T24, indicating weeks after initiation of treatment), and after discontinuation of treatment (T36 and T48, indicating weeks after initiation of treatment), normalized to average BTK expression in total B cells from healthy controls (set to 1). Data at left are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent minimum and maximum values. Graphs at right show the same patients at T0, T24, and T48. MFI = mean fluorescence intensity.

T cell infiltration in salivary glands is an important feature of pSS. Strikingly, BTK expression levels in total peripheral B cells correlated significantly with numbers of infiltrating $CD3^+$ T cells/mm^2 in parotid gland infiltrates (Figure 5E). These findings link BTK expression levels to autoantibody levels and salivary gland immune cell infiltration.

Abatacept treatment normalizes BTK expression in B cells from patients with pSS

Next, we wanted to investigate whether the increase of BTK expression levels in autoimmunity was dependent on T cell activation. To this end, we examined our 15 patients with pSS who had been treated for 24 weeks with the CTLA-4Ig fusion protein abatacept in the previously reported ASAP study [38], which was aimed at inhibiting T cell activity. Abatacept significantly reduced objective and subjective indicators of disease activity. Scores on the ESSDAI and EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) [44] as well as levels of RF and IgG decreased significantly, and health-related quality of life improved during abatacept treatment [38]. Interestingly, abatacept treatment significantly restored BTK expression levels in $CD27^-IgD^+$ naïve B cells, $CD27^+IgD^+$ and $CD27^+IgD^-IgM^+$ nonswitched memory B cells, and $CD27^+IgD^-IgM^-$ switched memory B cells to levels comparable to those in healthy individuals (Figure 6). Taken together, these findings show that targeting T cell activation in patients with pSS by abatacept restored BTK protein expression in B cells to normal levels after 24 weeks of treatment and suggest the involvement of a B cell- and T cell-driven proinflammatory loop in pSS.

DISCUSSION

We previously found in CD19-hBTK-transgenic mice that increased BTK protein levels in B cells are sufficient to disrupt T cell homeostasis and to establish systemic autoimmunity. Therefore, we decided to investigate whether BTK-driven proinflammatory loops may also propagate the development of autoimmune disease in humans. Upon B cell activation, only a small fraction of the BTK molecules present in a cell become detectably phosphorylated in a transient manner. Therefore, in this study, we made use of the unique property of BTK that its protein expression levels are stably up-regulated upon B cell activation [28], allowing for very sensitive measurements of changes in BTK signaling by intracellular flow cytometry. Importantly, BTK protein expression levels correlated with phosphorylation of BTK at Y^{551}. In the present study, we show that BTK expression is up-regulated upon BCR stimulation *in vitro* and that BTK is differentially expressed in human B cell subsets *ex vivo*. Notably, in healthy controls, *ex vivo* BTK expression is significantly increased in $CD27^+IgD^-IgM^+$ and $CD27^+IgD^+$ nonswitched memory B cells

compared to $CD27^{-}IgD^{+}$ naïve B cells and $CD27^{+}IgD^{-}IgM^{-}$ switched memory B cells. These findings suggest a role for BTK protein up-regulation in early activation of B cells prior to or during the GC reaction.

BTK protein and phospho-BTK were significantly increased in B cells from ACPA-positive RA patients but not in those from ACPA-negative RA patients. BTK was increased both in naive and in memory B cells and correlated with frequencies of circulating $CCR6^{+}$ Th17 cells. As ACPA positivity is associated with a more severe disease course, our findings point towards a pathogenic role of BTK-mediated signaling in RA. Likewise, BTK protein was increased in naive and memory B cells from a major fraction (~62%) of patients with pSS and correlated with serum RF levels and parotid gland T cell infiltration. Our data suggest that there were 2 subgroups of patients with pSS, based on BTK expression levels. Although BTK levels correlated with autoantibody levels in serum, they did not show a clear distinction in disease severity (the ESSDAI score) in our cohort of 26 patients. In addition, BTK levels in patients expressing low levels of BTK at baseline decreased upon treatment with abatacept in a manner similar to that in patients expressing high levels of BTK at baseline. Therefore, from the present study, we cannot conclude that these 2 subgroups of patients with pSS represent distinct subsets of patients, except for differences in BTK and autoantibody levels and T cell infiltrates in salivary glands. Analysis of a larger group of patients is needed to draw conclusions about the relationship between BTK expression and disease severity or frequencies of Th17 cells, and about the relationship between different clinical parameters.

We found increased BTK levels in subsets of patients with two distinct autoimmune diseases, indicating that disrupted BTK expression is not a unique feature of one disease but may be involved in more autoimmune diseases. Both RA and pSS are systemic autoimmune diseases, but B cells and autoantibodies have also been implicated in nonsystemic autoimmune diseases. Our data show a clear link between high BTK expression and autoantibodies, suggesting that autoimmune diseases featuring autoantibody production may be interesting candidates for future studies.

Several of our findings point to an association of BTK activity in B cells with T cell activation. These include the observed correlations of BTK expression levels with the frequency of circulating Th17 cells and ICOS expression on Tfh cells in RA patients, and with salivary gland T cell infiltration in pSS. In addition, the specific increase in BTK activity in ACPA-positive RA patients but not in ACPA-negative RA patients may require the involvement of T cells, since ACPA production is very likely T cell-dependent [45–47]. Interestingly, targeting T cell activation in patients with pSS by treatment with abatacept restored BTK protein expression in B cells to normal levels, which suggests that increased BTK expression in circulating B cells of patients with pSS depends on T cell activity. However, it is quite difficult to explain how abatacept treatment would reduce BTK expression in naive circulating B cells.

A direct effect of abatacept on naive B cells cannot be excluded, since it has been reported that expression of CD86 on B cells, even on naive B cells, is increased in patients with various autoimmune diseases [48,49], and we show in the present study that increased CD86 expression on naive B cells is correlated with higher BTK expression. Nevertheless, our data show that in patients with pSS, BTK expression is even increased in naive resting $CD86^-$ B cells, indicating that elevated BTK levels do not simply reflect the massive B cell activation in these patients. In addition, B cell-T cell interaction through costimulatory molecules [50] and/or T cell-derived cytokines may also affect naive B cells. The observed correlation between BTK levels in peripheral blood B cells and parotid gland T cell infiltration suggests that at an early stage of disease, either T cell activity regulates the expression of BTK in the B cell lineage or elevated BTK levels contribute to local T cell activity. These two scenarios are not mutually exclusive and are supported by our findings in BTK-overexpressing mice, in which the spontaneous autoimmune phenotype is dependent on a B cell-T cell-mediated proinflammatory feedback loop through CD40-CD40L interaction [28,33]. Further experiments are needed to reveal the molecular mechanisms that regulate BTK protein expression in naive B cells in patients with autoimmune disease.

Recent studies have pointed toward a pathogenic role for BTK signaling in autoimmune disease. In B cells from RA patients, BTK signaling was required for induction of interleukin-21 (IL-21) signaling by B cells [51]. Even though levels of phospho-Btk were not significantly different between RF-negative and RF-positive RA patients, there was a significant correlation between phospho-BTK and RF titer. In addition, disease activity in SLE was shown to correlate with expression in peripheral blood B cells of the transcription factor AT-rich-interactive domain-containing protein 3A [52], which interacts directly with BTK [53]. It was recently reported that levels of phospho-Syk, an upstream activator of BTK that has the capacity to phosphorylate BTK at position Y^{551} [18], were higher in peripheral blood B cells from RA patients, particularly those who were ACPA positive [54]. Those authors also found that treatment with abatacept significantly reduced the levels of phospho-Syk, but it is not known whether phospho-Syk levels are also modulated between different B cell subsets, similar to BTK. In this context, it is remarkable that increased phospho-Syk was found both in treatment-naive RA patients and in patients receiving methotrexate or methotrexate and biologic agents [54]. A recent meta-analysis concluded that the Syk inhibitior fostamatinib has moderate effects in the treatment of RA, with mostly mild-to-moderate adverse events and dose-dependent, transient neutropenia and hypertransaminasemia [55].

In summary, both naive and nonswitched memory B cells in peripheral blood from RA patients and a major fraction of patients with pSS showed significantly increased BTK protein levels, which correlated with ACPA positivity and severity of salivary gland T cell infiltration, respectively. Conversely, in CD19-hBTK-transgenic mice, we have noticed

that BTK overexpression alone is sufficient to disrupt T cell homeostasis and induce Tfh cell formation [28,33]. Furthermore, CD19-hBTK-transgenic mouse B cells show higher production of proinflammatory cytokines IL-6 and interferon-γ, which was dependent on T cells [33]. Together, these findings point to a BTK-dependent proinflammatory feedback loop whereby B cell-T cell interactions through costimulatory molecules and proinflammatory cytokine production drive autoimmunity. Therefore, by interfering with the costimulatory pathways between T cells and CD80/CD86-expressing dendritic cells or activated B cells, abatacept treatment may disrupt this feedback loop. In this context, it is important that it has been shown that strong CD28 engagement to CD86 is crucial for generating the Tfh cells that support GC development [56]. It is therefore attractive to speculate that in autoimmune disease, BTK-mediated signaling in B cells may establish or maintain T cell-propagated pathology and vice versa. Together with the observed beneficial effects of BTK inhibition in mouse models for autoimmune disease and its compelling safety and efficacy in patients with B cell malignancies [18,19], our findings would make BTK an attractive therapeutic target in autoimmune diseases, but we will have to await results of the ongoing clinical trials.

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SUPPLEMENTARY MATERIALS

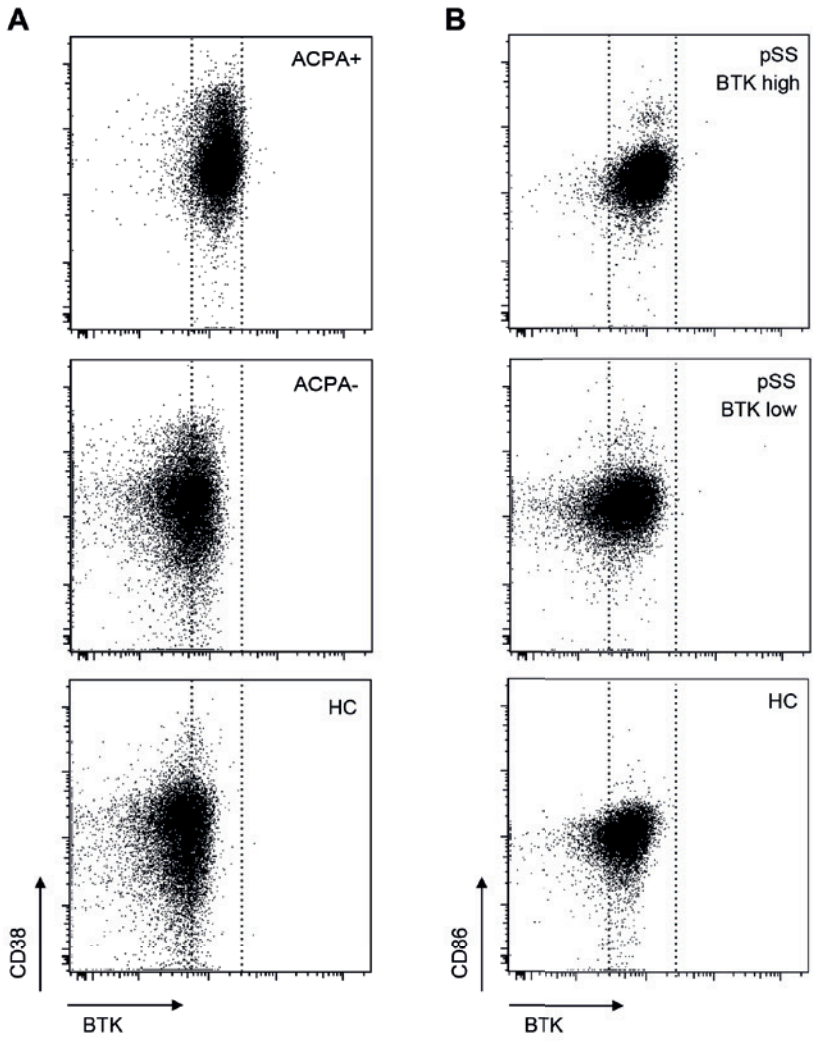
SUPPLEMENTARY TABLE 1 | Patients characteristics

	HC (n=15)	ACPA$^+$ RA (n=15)	ACPA$^-$ RA (n=15)
male / female	4 / 11	4 / 11	4 / 11
age in years (range)	52 (37-80)	47 (35-75)	53 (37-82)
disease duration in months (range)	-	6 (1-12)	3 (0-11)
DAS44 score (range)	-	3.0 (1.5-4.6)	2.9 (1.5-4.1)
ACPA in IU/mL (range)	-	454 (14-1802)	-
	HC (n=26)	**pSS (n=11)**	**pSS ASAP (n=15)**
male / female	4 / 22	0 / 11	3 / 12
age in years (range)	47 (22-72)	47 (21-70)	43 (18-68)
disease duration in months (range)	-	60 (5-133)	11 (1-156)
ESSDAI score (range)	-	11 (4-40)	10 (2-21)
Anti-Ro52 in AU/mL (range)	-	5501 (661-17032)	2580 (32-26430)
Anti-Ro60 in AU/mL (range)	-	7308 (18-29084)	10167 (255-73958)

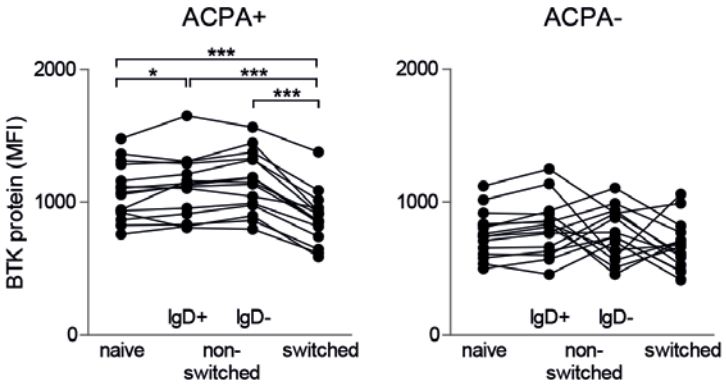
Continuous data are presented as median (range). HC = healthy controls, pSS = primary Sjögren's syndrome, RA = rheumatoid arthritis, ASAP = Active Sjögren Abatacept Pilot; DAS44 = Disease Activity Score in 44 joints; ESSDAI = EULAR Sjögren's Syndrome Disease Activity Index.

SUPPLEMENTARY TABLE 2

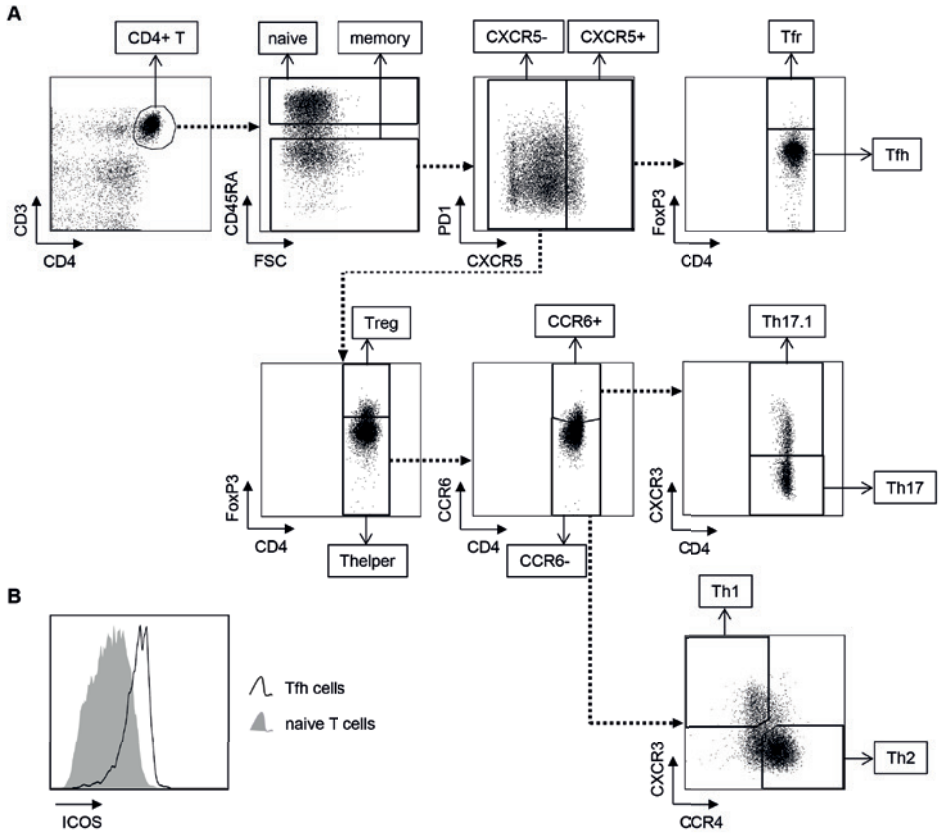
Specificity	Clone	Company
B cell stainings		
CD3	UCHT1	eBiosciences
CD19	SJ25C1	BD Biosciences
CD27	MT272	BD Biosciences
IgD	IA6-2	BD Biosciences
IgM	G20-127	BD Biosciences
BTK	53/BTK	BD Biosciences
T cell stainings		
CD3	UCHT1	eBiosciences
CD4	OKT4	eBiosciences
CD45RA	HI100	BD Biosciences
CXCR3	1C6/CXCR3	BD Biosciences
CCR4	205410	R&D
CXCR5	MU5UBEE	eBiosciences
CCR6	11A9	BD Biosciences
FoxP3	236/E7	eBiosciences
PDL-1	EH12.1	BD Biosciences
ICOS	ISA-3	eBiosciences
Phosphoflow		
CD3	UCHT1	BD Biosciences
CD19	HIB19	BD Biosciences
pBTK	pY551/ITK	BD Biosciences



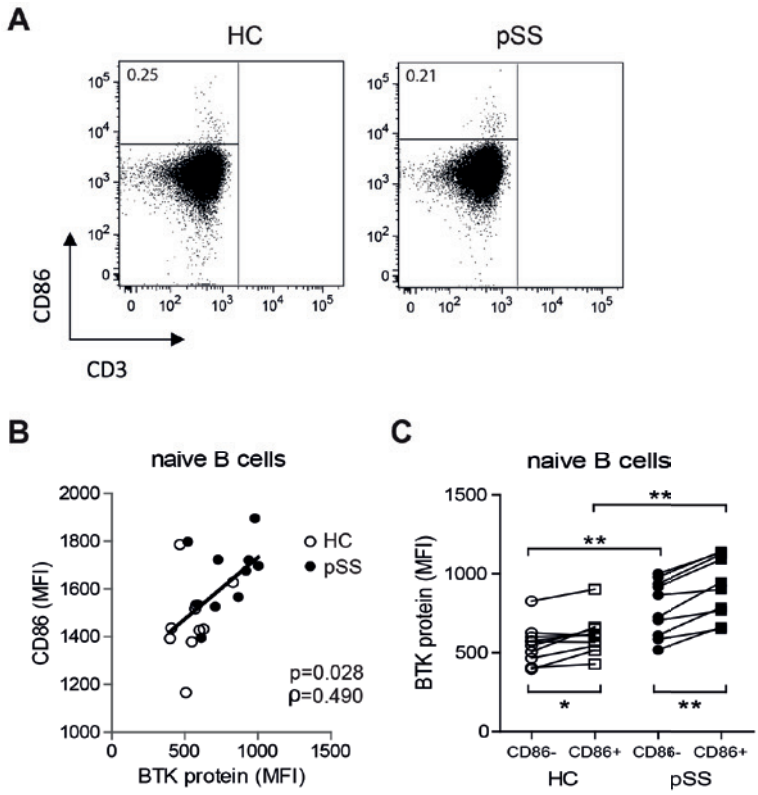
SUPPLEMENTARY FIGURE 1 | Representative flow plots of total circulating B cells of an ACPA+ and ACPA- RA patient and a healthy control (HC) (**A**) and a Btk high and BTK low pSS patient and a HC (**B**). Dotted lines indicate the location of the ACPA+ RA (**A**) or BTK high pSS (**B**) B cell population in the flow plots.



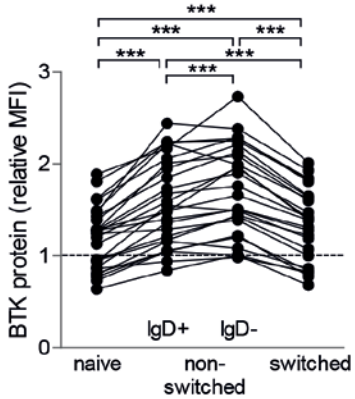
SUPPLEMENTARY FIGURE 2 | BTK protein expression in indicated B cell subsets of ACPA+ and ACPA- patients, analyzed by flow cytometry.



SUPPLEMENTARY FIGURE 3 | A, Gating strategy for T cell subsets. **B**, Representative graph of ICOS expression on naive T cells and Tfh cells.



SUPPLEMENTARY FIGURE 4 | **A**, Representative flow plots of CD86- and CD86+ naive B cells (CD19+IgD+CD27-). **B**, Correlation of BTK protein expression levels with CD86 expression on naive B cells in pSS patients and healthy controls. **C**, BTK protein expression levels in CD86- and CD86+ naive B cells from pSS patients and healthy controls.



SUPPLEMENTARY FIGURE 5 | Bruton's tyrosine kinase (BTK) protein expression levels, as measured by mean fluorescence intensity (MFI) in indicated B cell subsets of 26 pSS patients. Symbols represent individual subjects. Dashed line indicates average MFI in total B cells. *** P value < 0.001.

10

SUMMARY AND GENERAL DISCUSSION

SUMMARY

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disease, characterized by lymphocytic infiltration of exocrine glands, and the salivary and lacrimal glands in particular. Predominant symptoms of pSS are a sensation of dry mouth, dry eyes, and chronic fatigue. In addition to the exocrine glands, many other organs can be affected by the disease as well, emphasizing the systemic nature of pSS. Hyperactivity of B lymphocytes is thought to play a central role in the pathogenesis of pSS. Therefore, these cells are considered to be an important target for treatment. More recently, a pathogenic role for T cells has also been recognized, which includes their helper function to (autoreactive) B cells, amongst others. The research described in this thesis addresses two aims: (1) to examine T cell and B cell-related biomarkers of pSS and (2) to assess the effect of rituximab and abatacept treatment on T cell-dependent B cell hyperactivity in pSS patients.

In the first part of this thesis, the relevance of several T cell and B cell-related biomarkers of pSS is described. We evaluated their capacity to predict and/or monitor disease initiation, clinical manifestations, and/or disease progression. In **chapter 2** we reviewed the current knowledge on pathogenicity and plasticity of Th17 cells in pSS. We concluded that Th17 cells/IL-17 producing T cells are involved in local inflammation in pSS, via pro-inflammatory effects on salivary gland epithelial cells and support of ectopic lymphoid tissue formation. The contribution of Th17 cells to systemic disease activity remains, however, more enigmatic. The latter might be a consequence of plasticity of this cell subset. We postulated that plasticity towards Th17.1 cells, co-expressing IL-17 and IFN-γ, may support chronic inflammation and B cell activation in pSS patients.

In addition to Th17 cells, a more dedicated subset of B cell helper T cells, named T follicular helper (Tfh) cells, has been identified. In **chapter 3a** and **3b** we described studies assessing the prevalence and phenotype of circulating Tfh (cTfh) cells and their regulatory counterparts, i.e. T follicular regulatory (Tfr) cells, in pSS patients and controls. We showed that frequencies of both subsets were increased in blood from pSS patients compared to healthy controls. Circulating Tfr (cTfr) cells were even further increased than cTfh cells, resulting in significantly higher cTfr/cTfh ratios in pSS patients, compared with either healthy controls (**chapter 3a**) or non-SS sicca controls (**chapter 3b**). Frequencies of cTfh cells and cTfr cells correlated with serum levels of IgG and CXCL13, and with systemic disease activity, as measured by the EULAR Sjögren's syndrome disease activity index (ESSDAI) and clinical ESSDAI. These results indicate that cTfh and cTfr cell frequencies are useful biomarkers of systemic disease activity in pSS. The positive correlation between cTfr cell frequencies, B cell hyperactivity and systemic disease activity is, however, remarkable, as Tfr cells are supposed to suppress humoral immune responses. A possible explanation for a reduced suppressive capacity of Tfr

cells in pSS comes from our finding that circulating Treg cells, and in particular cTfr cells, from pSS patients express decreased levels of the inhibitory receptor CTLA-4.

The next two chapters focused on B cell-related biomarkers of pSS. In the study described in **chapter 4** we aimed to characterize a subset of epithelium-associated B cells expressing Fc-receptor-like protein 4 (FcRL4). The presence of FcRL4$^+$ B cells around and within the ductal epithelium of inflamed glandular tissue can be seen as a histologic hallmark of pSS. Furthermore, these cells are possibly precursor cells of mucosa-associated lymphoid tissue (MALT) lymphoma, a type of B cell lymphoma that occurs in 5-10% of pSS patients, preferentially in the parotid glands. Because FcRL4 is widely expressed by MALT lymphomas, the presence of large numbers of non-neoplastic FcRL4$^+$ B cells in parotid gland tissue may identify patients who are at risk of lymphoma development. For the purpose of characterization, we isolated 'normal' FcRL4$^+$ B cells from parotid gland tissue of pSS patients without MALT lymphoma and performed single cell RNA sequencing. We found that FcRL4$^+$ B cells from parotid glands of pSS patients showed upregulation of genes involved in homing and cell adhesion, consistent with their tissue location close to the epithelium. FcRL4$^+$ B cells also showed upregulation of genes that promote inflammation and B cell survival. We postulated that these cells contribute significantly to the epithelial damage seen in the glandular tissue of pSS patients, and that these cells are prone to lymphomagenesis.

Following up on identification of potential biomarkers of MALT lymphoma, we described in **chapter 5** that 50% of pSS patients with salivary gland MALT lymphoma had an aberrant ratio of serum immunoglobulin free light chains (FLC), with a relative increase in FLCκ compared to FLCλ. In pSS patients without MALT lymphoma, levels of both FLCκ and FLCλ were often increased, but abnormal ratios were rarely seen. We concluded that the FLC κ/λ ratio is a useful biomarker of MALT lymphoma presence, which can be used in combination with conventional biomarkers such as cryoglobulinemia, lymphopenia, low complement levels, and persistent parotid gland enlargement. In this chapter we also showed that serum levels of FLCκ, and to a lesser extent FLCλ, can be used to monitor the effect of immunomodulatory treatment on B cell activity in pSS patients.

In the second part of this thesis we assessed the effect of rituximab and abatacept treatment on T cell-dependent B cell hyperactivity in pSS patients. In **chapter 6** we described a study in which we showed that B cell depletion therapy with rituximab had significant effects on the T cell compartment, in addition to the well-described effects on the B cell compartment. Among T cells, in particular cTfh cells were affected and frequencies of these cells were normalized to levels seen in healthy controls. The reduction in cTfh cells was associated with improved objective clinical disease activity measures. In **chapter 7** we summarized and discussed current literature on clinical

and biological effects of rituximab treatment in pSS. We concluded that rituximab has beneficial effects on B cell activity, glandular morphology, dryness, fatigue and several extraglandular manifestations in at least subgroups of pSS patients. Available evidence suggested that patients with moderate to severe systemic involvement, i.e. activity in multiple ESSDAI domains, may benefit most from treatment. In addition to B cell-targeting therapies, abatacept treatment (aiming at inhibition of T cell activation) also showed beneficial clinical effects in pSS patients. In **chapter 8** we described the effect of this treatment on T cell homeostasis and T cell-dependent B cell hyperactivity in pSS. Abatacept treatment reduced numbers of cTfh cells, as well as expression of the activation marker ICOS on T cells, both in the periphery and locally in parotid gland tissue. The decrease in ICOS expression on the remaining cTfh cells was significantly associated with the reduction in ESSDAI scores over time. B cell hyperactivity was also decreased by abatacept treatment, as reflected by lower levels of circulating plasmablasts and autoantibody titers.

Finally, we showed in the study described in **chapter 9** that abatacept treatment resulted in decreased expression levels of Bruton's tyrosine kinase (BTK) in B cells from pSS patients. BTK is a signaling molecule that directly links B cell receptor (BCR) signals to B cell proliferation and survival. At baseline, BTK protein expression was increased in a majority of pSS patients, and correlated with serum rheumatoid factor levels and parotid gland T cell infiltration. Together with the findings described in **chapter 8**, these observations illustrate the pivotal role of the crosstalk between B cells and T(fh) cells in the pathogenesis of pSS.

GENERAL DISUSSION

To date, treatment options for primary Sjögren's syndrome (pSS) are still symptomatic. Although several immunomodulatory treatments show promising clinical and biological outcomes, heterogeneity in clinical signs and immune activation patterns between patients hampers successful drug development and registration. To address this issue, biomarkers that enable stratification of clinical and molecular phenotypes and identification of new, (patient-)specific, targets for treatment are urgently needed. In this thesis we aimed to evaluate new biomarkers and treatment targets by elucidating the role of T cell-dependent B cell hyperactivity in the pathogenesis of pSS. This chapter discusses the key findings of this thesis and identifies areas for future research.

Part I. T cell-dependent B cell hyperactivity: Biomarker of disease?

After the Th1/Th2 paradigm for adaptive immunity was challenged, the role of newly recognized effector subsets, including Th17 cells and T follicular helper (Tfh) cells, gained much attention. In the past decade, these new subsets have been extensively studied, in particular in the context of inflammatory diseases and autoimmunity [1,2]. $CD4^+$ effector cell subsets can be discriminated based on chemokine receptor expression patterns and/or cytokine producing capabilities. However, gradually it became clear that $CD4^+$ T cell effector subsets are not necessarily committed to a single differentiation fate, but that certain subsets show plasticity, i.e. the ability to adapt different effector functions [3]. For example, $CD4^+$ T cells that co-express IL-17 and IFN-γ (named Th17.1 cells) have been associated with chronic inflammation [4]. Tfh cells also come in different phenotypes, and can be sub-divided in Tfh1, Tfh2, and Tfh17 cells, based on expression patterns of CXCR3 (associated with Th1 cells) and CCR6 (associated with Th17 cells) [5].

Th17 cells in pSS

Th17 cells and their signature cytokine IL-17 are present in inflamed salivary glands of pSS patients [6–8]. In the glands they may contribute to the disease by activation of epithelial cells and stimulation of ectopic lymphoid tissue formation (reviewed in **chapter 2**). The current literature demonstrates that Th17 cells play a crucial role in initiation and progression of disease in several mouse models of SS [9–11]. However, the contribution of Th17 cells to human pSS in ambiguous, which may be a result of significant plasticity as well as phenotypic heterogeneity of this cell subset. To complicate things further, the definition of Th17 cells by either cytokine production or chemokine receptor expression is still a matter of debate. We proposed in **chapter 2** that local differentiation of Th17 cells towards Th17.1 cells, co-expressing IL-17 and IFN-γ, contributes to chronic inflammation and B cell activation in the inflamed glands.

However, addressing the fate and functionalities of infiltrated T cells in the inflamed exocrine glands of pSS patients remains a challenge, because of limited availability of fresh biopsy material, especially at different time points of disease development, and changes in environmental cues when cells are isolated for *in vitro* fate-mapping and functional studies.

Tfh cells and T follicular regulatory cells in pSS

Although the necessity of T cell help for antibody responses was described decades ago, the recognition of a dedicated subset of B cell helper T cells, named Tfh cells, followed much later. First, the chemokine receptor CXCR5, promoting migration to B cell follicles, was linked to Tfh cells [12]. Other key molecules such as BCL6, IL-21, PD-1 and ICOS were subsequently revealed. However, no single marker or combination of markers can unequivocally identify Tfh cells, as expression of Tfh cell-related molecules is dynamic and heterogeneous [2]. Tfh cells facilitate B cell activation, and increased numbers of Tfh cells have been associated with several B cell-mediated autoimmune diseases, including pSS [13]. We found in several study cohorts that frequencies of circulating Tfh cells, defined as $CD4^+CD45RA^-CXCR5^+PD\text{-}1^+$ cells, were increased in pSS patients compared with healthy individuals (see **chapters 3a, 6 & 8**). This increase is already present at the time of diagnosis, and Tfh cell frequencies correlated with serum IgG levels and systemic disease activity scores, as measured by EULAR Sjögren's syndrome disease activity index (ESSDAI) and clinical ESSDAI (clinESSDAI: ESSDAI without the biological domain[14]). Circulating Tfh cells are therefore a useful biomarker of B cell hyperactivity and systemic disease activity in pSS, and can be used to monitor extraglandular involvement in pSS patients over time.

Recently, a regulatory subset of Tfh cells, named T follicular regulatory (Tfr) cells, has been identified [15]. These cells are able to control Tfh cell proliferation and consequently B cell activation (reviewed by [16]). **Chapters 3a** and **3b** of this thesis described the frequency and phenotype of circulating Tfr (cTfr) cells in a large group of pSS patients. In addition to cTfh cells, frequencies of cTfr cells were elevated in pSS patients compared with healthy individuals. Furthermore, increased expression of the chemokine receptor CXCR3 was observed on cTfh and cTfr cells from pSS patients. Expression of CXCR3 enables migration from the circulation towards inflamed glandular tissues where CXCL10, an important ligand for CXCR3, is produced [17]. The importance of Tfr cells for regulation of antigen-specific immune responses was recently illustrated in a *Bcl6$^{fl/fl}$Foxp3Cre/Cre* mouse model, in which Tfr cells were diminished [18]. When this mouse model was combined with an experimental Sjögren's syndrome (ESS) model, in which mice are immunized with salivary gland proteins, disease started earlier and worsened. Tfr-deficient mice showed enhanced serum levels of autoantibodies against

salivary gland proteins and increased frequencies of germinal center (GC) B cells in the cervical lymph nodes. On the other hand, when a knock-out model for Tfh cells (*Bcl6$^{fl/fl}$Cd4Cre*) was combined with the ESS model, mice were protected from lymphocytic infiltration, excessive GC responses and autoantibody production, while salivary flow was not greatly improved [18]. Together, these results underline the importance of Tfr/Tfh cell balance in protecting mice from autoimmune disease. However, these cells do not seem to contribute significantly to the impairment of saliva production in the ESS model. Similarly, the relation between local immune responses and hyposalivation in human pSS remains unclear, and there is a relatively weak association between saliva production and the degree of salivary gland inflammation [19]. While a lack of Tfr cells can exacerbate autoimmune disease in mice, we showed in **chapters 3a** and **3b** that cTfr cells in pSS patients were even further increased than cTfh cells, resulting in a significantly higher cTfr/cTfh ratio. An increased Tfr/Tfh ratio in pSS patients was also recently described by Fonseca et al. [20,21]. Of note, in their studies Tfr cells and Tfh cells were defined by expression of CXCR5 only. We included CXCR5^{+}PD-1^{+} cells, because the gene expression profile of circulating PD-1^{+} memory Tfh is more polarized towards Tfh cells, and these cells exhibit a greater B helper capacity compared to CXCR5^{+}PD-1^{-} cells [22].

Whereas an expanded regulatory cell population would suggest increased immune suppression, this is clearly not the case in pSS. We reported in **chapter 3a** that cTfr cell frequencies correlate positively with serological markers of B cell activity and systemic disease activity. Interestingly, measurement of CTLA-4 expression in cTfr cells showed that levels of this inhibitory receptor are significantly lower in pSS patients, compared with healthy individuals. CTLA-4 is a critical receptor that mediates suppression of humoral immune responses by regulatory T cells [23,24]. The importance of this receptor in immune homeostasis is illustrated by the finding that CTLA-4-deficient mice die from T cell-dependent multi-organ infiltration [25,26]. Mutations in the *CTLA4* gene in humans that result in haploinsufficiency were associated with a complex dominant immune dysregulation syndrome, with clinical features that are related to autoimmunity (e.g., cytopenia) as well as immunodeficiency (e.g., recurrent infections) [27]. Decreased expression of CTLA-4 by Tfr cells in pSS may -at least partially- explain why control of Tfh cell expansion and B cell responses in pSS are impaired (Figure 1). Our data reinforce the need for additional functional studies to assess suppressive capacity by regulatory T cells in this disease.

Epithelium-associated B cells in pSS

A characteristic histopathological finding in salivary gland lesions of pSS patients is infiltration of B cells, located in close proximity to, or even within, the ductal epithelium. A substantial proportion of the intra-epithelial B cells express Fc receptor-like protein

4 (FcRL4) [28], and these cells seem to contribute significantly to the formation of lymphoepithelial lesions (LELs). FcRL4 is an inhibitory receptor that can bind IgA and is typically expressed by B cells residing in mucosa-associated lymphoid tissue (MALT) [29,30]. Binding of IgA possibly functions as a negative feedback mechanism to control formation of IgA-producing plasma cells. Furthermore, FcRL4$^+$ B cells in the salivary glands of pSS patients may serve as precursor cells of salivary gland MALT lymphoma, as FcRL4 is widely expressed by these lymphomas [29]. To investigate if the presence of FcRL4$^+$ B cells can act as a biomarker to identify patients at risk of MALT lymphoma development, additional knowledge on the origin, function and fate of FcRL4$^+$ B cells is necessary.

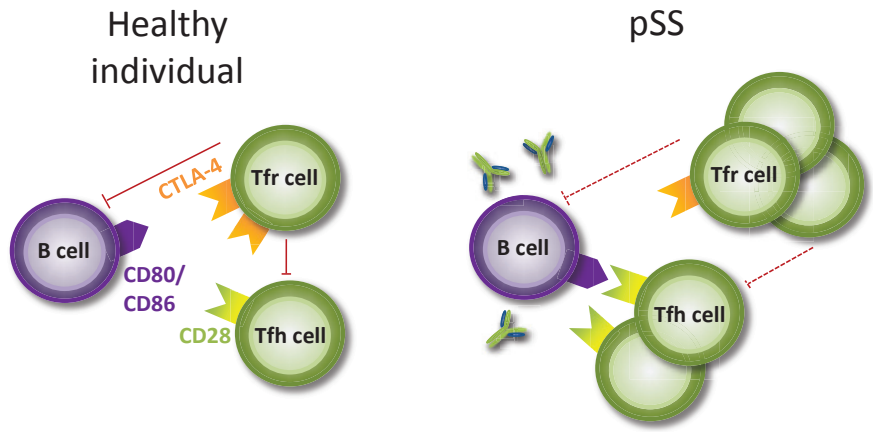


FIGURE 1 | Reduced immune suppression by T follicular regulatory cells in patients with primary Sjögren's syndrome. In healthy individuals, T follicular regulatory (Tfr) cells suppress activation of B cells and T follicular helper (Tfh) cells through CTLA-4. In pSS patients, frequencies of circulating Tfr cells are increased, while expression levels of CTLA-4 by these cells are decreased. Consequently, suppression of B cell responses as well as Tfh cell proliferation by Tfr cells in pSS patients may be impaired. CTLA-4: cytotoxic T-lymphocyte–associated antigen 4.

In **chapter 4** the prevalence and phenotype of circulating FcRL4$^+$ B cells in pSS patients and non-SS sicca patients was assessed to explore the possibility that these cells are increasingly activated at mucosal tissue sites and then migrate, via the blood, to the inflamed salivary glands. We observed, however, no difference in the frequency of circulating FcRL4$^+$ B cells between pSS patients and non-SS sicca patients, and the prevalence was generally low (±0.5% of B cells). An alternative possibility could be that FcRL4 is locally upregulated by infiltrated B cells upon stimulation by epithelial cells, T cells or other environmental triggers. This hypothesis is supported by the finding that stimulation of 'healthy' human memory B cells with CD40L and a TLR9-agonist induces FcRL4 expression by the majority of memory B cells [31].

To characterize local FcRL4$^+$ B cells in pSS, these cells were isolated from parotid gland tissue of patients and single-cell RNA sequencing was performed. Sequencing results revealed that the transcriptional profile of FcRL4$^+$ B cells is more similar to FcRL4$^-$CD27$^+$ 'memory' cells, than FcRL4$^-$CD27$^-$ 'naive' cells. Consistent with the phenotype that we observed in blood, increased transcript levels of *CXCR3* were found in glandular FcRL4$^+$ B cells. As mentioned before, expression of CXCR3 may facilitate migration to inflamed salivary gland tissue, and specifically to the ductal epithelium, where its ligand CXCL10 is produced. Additionally, compared with FcRL4-negative B cells, FcRL4$^+$ B cells showed significant upregulation of integrins, the NFκB-pathway and pro-survival factors including the BAFF/APRIL receptor TACI. The upregulation of integrins, including CD11c, by FcRL4$^+$ B cells may explain their retention within the epithelium. Upregulation of the NFκB-pathway could be a result of increased ligation of TACI by BAFF and/or APRIL [32]. Ligation of TACI can also result in AID induction, and consequently isotype switching or somatic hypermutation [33]. In line with this notion, sequencing data from single cells revealed that transcript levels of AID were enriched in FcRL4$^+$ B cells. Of note, histopathological analysis of the corresponding diagnostic biopsies of the included patients showed that the infiltrates did not harbor GCs, based on the H&E staining. Together, the results described in **chapter 4** suggest that glandular FcRL4$^+$ B cells in pSS are chronically activated, pro-inflammatory B cells, which may undergo isotype switching and/or somatic hypermutation at extrafollicular sites. The expression of AID, together with a high proliferation rate and expression of pro-survival factors by FcRL4$^+$ B cells in salivary gland tissues of pSS patients may put these cells at risk of mutagenesis. A genetic predisposition, for example a polymorphism of *TNFAIP3* (A20: a protein that inhibits NFκB signaling) [34], could be an additional risk factor. As a consequence, pSS patients who harbor FcRL4$^+$ B cells in their salivary glands may be at risk of MALT lymphoma development. The finding that more FcRL4$^+$ B cells are present in parotid glands, compared to labial glands, may explain why MALT lymphomas in pSS patients preferentially develop in parotid glands [28]. Although histology is the gold standard to confirm a diagnosis of MALT lymphoma, taking a biopsy is invasive and progression cannot be monitored easily over time. Serological markers of MALT lymphoma may therefore aid in 1) identifying patients at risk, and 2) monitoring disease progression and response to treatment in daily clinical practice.

Serological markers of B cell hyperactivity

The central role of B cell hyperactivity in pSS pathogenesis is widely recognized. In addition to conventional biomarkers of B cell activity such as serum levels of total IgG and autoantibodies, several other B cell-related markers have been investigated in serum of pSS patients. These include β2-microglobulin, BAFF, CXCL13 and immunoglobulin

free light chains (FLC) [35–37]. In **chapter 5**, serum levels of FLC in pSS and non-SS sicca patients are presented. FLC levels, and in particular FLCκ, correlated with systemic disease activity, as measured by ESSDAI as well as clinESSDAI. We further showed that the FLC κ/λ ratio is a potential biomarker of salivary gland MALT lymphoma, as 50% of the MALT lymphoma patients had an aberrant ratio. A recent study identified cryoglobulinemia, parotid gland enlargement and lymphadenopathy as strong predictors of MALT lymphoma presence in pSS [38]. However, in a previous study we reported that only 11% (4/35) of MALT lymphoma patients had cryoglobulinemia, indicating that this biomarker lacks sensitivity [39]. On the other hand, 77% (27/35) of MALT-pSS patients experienced parotid gland swelling [39], but this symptom is also frequent in pSS patients without lymphoma [40]. Our study showed that the κ/λ ratio might serve as a valuable additional biomarker to identify and monitor patients with MALT lymphoma. A larger, prospective study is needed to prove its predictive value in addition to previously recognized predictive factors, such as cryoglobulinemia and persistent parotid gland enlargement. In **chapter 5** we also presented longitudinal data of FLC levels in pSS patients before and after treatment with either rituximab or abatacept. The data reported in this chapter indicate that FLC levels are useful for monitoring the effect of treatment on B cell activity, because the FLC levels have a shorter half-life than IgG and are sensitive to change.

Part II. T cell-dependent B cell hyperactivity: Target for treatment?

Effects of rituximab treatment on T cell-dependent B cell hyperactivity

B cell depletion therapy with rituximab (anti-CD20) was one of the first biologic disease-modifying anti-rheumatic drugs (DMARD) that was clinically tested in pSS. Up to now, several studies have evaluated the efficacy of rituximab in pSS, with inconsistent outcomes (reviewed in **chapter 7**). To understand variability in clinical response between pSS patients, it is important to study the effects of treatment on the immune system. In **chapter 6** we assessed the effects of rituximab treatment on the T cell compartment of pSS patients. We hypothesized that depletion of B cells, and consequently inhibition of antigen presentation and cytokine production by these cells, would affect T cell activation. Indeed, numbers and frequencies of cTfh cells were significantly decreased during B cell depletion, and to a lesser extent also circulating Th17 cells were reduced. In addition, serum levels of IL-21 and IL-17 were significantly lowered by treatment. Importantly, the decrease in cTfh cells correlated with the decrease in ESSDAI scores during B cell depletion. Numbers and frequencies of Th1 cells and Th2 cells were unaffected by treatment.

The specific effects observed on cTfh cells and Th17 cells can be explained by lower availability of IL-6 due to the depletion of B cells. This cytokine is involved in

the differentiation and activation of Tfh cells as well as Th17 cells [41]. Plasmablasts in particular produce high amounts of IL-6 [42]. A decrease in serum levels of IL-6 was indeed observed in a previous study [43]. Together, these results underline the importance of the IL-6/IL-21 axis in pSS pathogenesis. In addition to the observed effects on the T cell compartment, several other beneficial biologic effects of rituximab treatment in pSS have been shown (for a review see **chapter 7**). These effects include (partial) restoration of salivary gland morphology and reduction of autoantibody levels [44,45]. The restorative effects on salivary gland morphology involves the reduction in number and severity of the LELs, which seems to be a direct consequence of the depletion of $FcRL4^+$ B cells located within the epithelium [44]. These findings also illustrate the crosstalk between the $FcRL4^+$ B cells and the epithelium.

Effects of abatacept treatment on T cell-dependent B cell hyperactivity

Because $CD4^+$ T cells and B cells seem to act in a pro-inflammatory feedback loop in pSS patients, therapies that impair T cell activation are also feasible treatment options. Abatacept, a fully human fusion molecule combining cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) with IgG Fc, can bind to CD80/86 on antigen presenting cells (APC) and hereby inhibits T cell activation by these APCs. In **chapter 8** we studied the effects of abatacept treatment on T cells and B cells from treated pSS patients. We showed that specifically cTfh cells and peripheral Treg (pTreg) cells are reduced by treatment, and that the remaining cTfh cells express lower levels of inducible costimulator (ICOS), which is usually upregulated upon activation. The decrease in ICOS expression by cTfh cells was significantly associated with the decrease in ESSDAI scores during treatment. Importantly, protein levels of ICOS were also locally reduced in the inflamed salivary glands after 24 weeks of treatment. We did not specifically assess frequencies of cTfr cells in this study, but the decrease in cTfh cells as well as pTreg cells, which comprise cTfr cells, suggests that these cells are also reduced by abatacept treatment.

In addition to the observed effects on cTfh cells, pTreg cells, and ICOS expression, we showed that frequencies of circulating plasmablasts and serum levels of anti-SSA/Ro and anti-SSB/La were significantly decreased during treatment, which is likely a result of impaired differentiation of memory B cells into plasmablasts and short-lived plasma cells. The effects on cTfh cells and B cell activity were further reflected by the observed decrease in the number of GCs in parotid glands of treated patients [46]. Despite the observed effects on systemic and local B cell activity, total numbers of infiltrated T cells and B cells, and protein expression levels of IL-21 in parotid gland tissue, were not significantly affected by abatacept (**chapter 8** and [46]). Apparently, migration of lymphocytes into the inflamed tissue was not impaired, and IL-21 production was maintained.

Whether the number of Tfh cells in the glandular tissue is affected by abatacept treatment still needs to be investigated. However, also other $CD4^+$ T cell subsets may contribute to the production of IL-21 [47,48], and these cells may be activated via CD40-CD40L-mediated interaction with APCs, and/or as a result of continuous presence of IL-6 in the glands. Another explanation for continued local IL-21 production after abatacept treatment may come from a study in rheumatoid arthritis patients, showing that a subset of CD28-negative $CD4^+$ memory T cells infiltrated synovial tissues and maintained pro-inflammatory cytokine production [49]. Downregulation of CD28 by $CD4^+$ T cells may also occur in salivary gland tissue of pSS patients, and could influence the effectivity of costimulation blockade [50]. A third explanation for continued IL-21 production might be that mainly new formation of effector T cells is inhibited by abatacept and that turnover of the residing memory cells is relatively slow. A repeated biopsy after a longer period of treatment (e.g., one year) would be needed to address this issue. Lastly, we cannot exclude that drug penetration of salivary gland tissue is suboptimal compared to other tissues (e.g., synovial tissue and secondary lymphoid organs). Differences in drug penetration between tissues may be an additional explanation for the modest effects of abatacept treatment on salivary gland inflammation, while this drug can ameliorate multiple extraglandular manifestations of pSS.

The role of Bruton's tyrosine kinase in T-cell dependent B cell hyperactivity in pSS

Bruton's tyrosine kinase (BTK) is a key molecule involved in B cell receptor (BCR) signaling. In mice, overexpression of BTK in B cells results in a Sjögren/lupus-like autoimmune phenotype upon ageing, in a T cell-dependent manner [51,52]. Whether aberrant BTK levels were also involved in human autoimmunity was unclear. In **chapter 9** we showed that intracellular levels of BTK in B cells are increased in pSS patients and ACPA-positive rheumatoid arthritis patients. Although the highest increase was observed in memory B cells, also naive B cells from these patients showed increased BTK expression compared with healthy controls, indicating that this increase is not merely a result of chronic antigen exposure. In pSS patients, increased expression levels of BTK were associated with higher levels of RF and with higher numbers of infiltrated T cells in the parotid glands. The association with RF levels may be explained by a lower threshold for B cell activation when BTK expression, and consequently BCR signaling, is enhanced, resulting in enhanced plasma cell formation. A greater antigen-presenting potential by B cells as a result of higher BTK expression levels may explain the observed association with numbers of infiltrated T cells [53].

Interestingly, BTK expression levels in both naive and memory B cells were significantly decreased during abatacept treatment. This decrease could be a direct effect of abatacept on B cells via binding to CD80/86, as increased expression levels of CD86 on

naive B cells were associated with higher BTK expression. In addition, altered levels of T cell-derived cytokines during abatacept treatment may affect B cell activation and possibly also BTK expression levels, although such molecular mechanisms are relatively unexplored.

FUTURE PERSPECTIVES

Patient stratification by immune profiling

Because pSS is a heterogeneous disease, patient stratification could aid in the development of patient-tailored treatments. Potential biomarkers described in this thesis that can be used as a starting point for patient stratification are frequencies of Tfh cells and Tfr cells in blood, and possibly also BTK expression levels. Others have shown that the presence of an interferon (IFN) type I signature identifies a subgroup of pSS patients with high systemic disease activity and high levels of autoantibodies [54,55]. Whether the IFN signature represents a different pathologic mechanism, or whether it only indicates more active disease, remains to be shown [56].

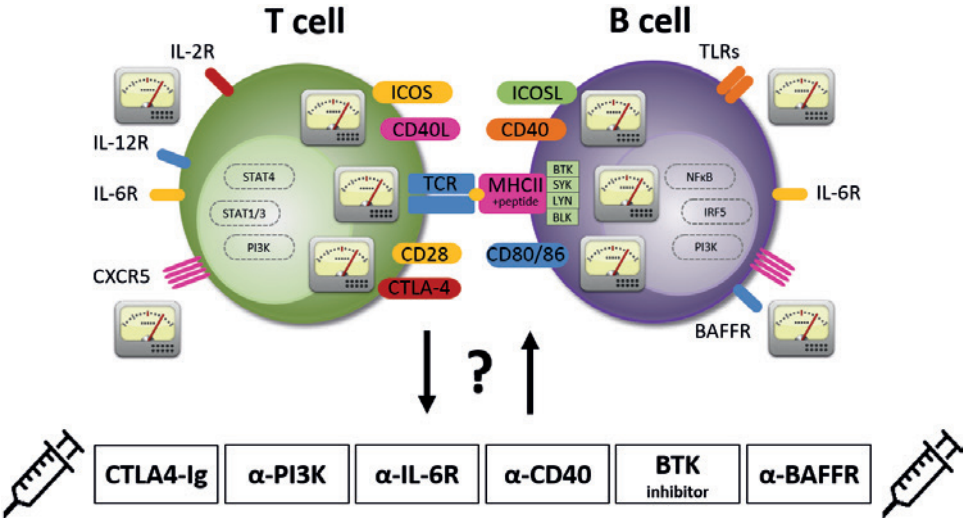


FIGURE 2 | Matching of patient-specific immune signaling and treatment. Critical immune signals, the sum of which determines T or B cell activation and expansion, are illustrated. Measuring the strength of each signal in an individual may aid in the establishment of patient-tailored treatment. Drugs that are currently under investigation in pSS target costimulatory pathways (CTLA4-Ig, α-CD40), cytokine signaling (α-IL-6R, α-BAFFR) or intracellular signaling (α-PI3K, BTK inhibitor).

So far, neither results from immunophenotyping studies nor genome wide association studies (GWAS) have facilitated patient stratification in pSS, and other approaches to identify patient-specific immune signals that contribute to pathogenesis

are needed. The majority of the currently available research, including the research presented in this thesis, assessed specific immune signals in large numbers of cells simultaneously and often on a patient group level. However, recent technologic advances such as (single cell) whole transcriptome sequencing have enabled us to measure an almost infinite number of immune signals at the same time. The amount of data generated by these techniques asks for a network-based approach. A potential way forward is to measure signaling differences in T cells and B cells from pSS patients on a single-cell and single-patient level (Figure 2). Such an approach enables detection of small, intrinsic changes in T cell and B cell signaling that are probably present in pSS patients, but still need to be unraveled. Subsequently, patient-specific immune profiles can be determined and personalized treatment with targeted biologicals can be established.

Promising treatments that target T cell-dependent B cell hyperactivity

Multiple biologic treatments that interfere with T cell-B cell interaction are currently under investigation in pSS. B cell-targeting therapies are still considered as potential treatment strategies, and the beneficial biologic effects that are seen after treatment of pSS patients with rituximab support further development of these therapies. In addition to rituximab, promising drug candidates that result in (partial) B cell depletion are epratuzumab (anti-CD22) and VAY736 (anti-BAFFR). The addition of epratuzumab to standard therapy in SLE patients did not result in higher response rates compared with placebo [57]. However, in a subgroup of anti-SSA-positive SLE patients with associated SS, response rates were higher in patients who received epratuzumab, compared to placebo [58]. Interestingly, these SLE patients with associated SS showed a faster and stronger B cell depletion compared to SLE patients without associated SS. Treatment with anti-BAFFR also resulted in B cell depletion [59], and clinical efficacy of this treatment in pSS is currently under investigation. An advantage of targeting BAFF-R is that in addition to B cell depletion, BAFF-BAFF-R signaling in the remaining B cells (i.e. plasmablasts and plasma cells) is inhibited. Intriguingly, while it is a non-depleting antibody, anti-CD40 treatment also showed promising effects in pSS patients by reducing ESSDAI scores significantly [60]. The main mechanism of action of anti-CD40 is probably inhibition of T cell-dependent B cell activation. However, CD40 can also be expressed by other cell types, including dendritic cells (DCs), and anti-CD40 may therefore exert additional B cell-independent effects, such as inhibition of T cell activation by DCs.

Other promising treatments for pSS that affect T cell-dependent B cell hyperactivity, without depletion of B cells, are abatacept (CTLA-4Ig), tocilizumab (anti-IL-6R), JAK1 inhibitors (e.g., filgotinib), and PI3Kδ inhibitors. Interestingly, these drug candidates may affect the formation of Tfh cells, as we have shown for abatacept. IL-6 is important for

differentiation of these cells, and JAK1 and PI3Kδ are involved in downstream signaling of IL-6R [61]. Lastly, BTK inhibitors are potential drug candidates to interfere with T cell-dependent B cell activation in pSS, in particular in patients with high BTK expression levels at baseline. Because BTK expression levels were associated with the amount of T cell infiltration in the inflamed glands of pSS patients, and also with frequencies of circulating Th17 cells in ACPA$^+$ RA patients, BTK inhibition may affect both T cells and B cells in the periphery and the affected tissues.

CONCLUDING REMARKS

Although no disease-modifying treatments for pSS patients exist to date, the understanding of the pathogenesis of pSS has increased significantly over the last decade. The use of new therapeutic options has contributed significantly to this increase in knowledge. Many promising therapies are currently under investigation and at least one of these agents tested will probably be approved in a not-too-distant future. We showed that Tfh cells are a useful biomarker and treatment target for pSS. We presume that interruption of T cell-B cell interaction, at either side, is crucial for successful treatment of systemic disease activity in this disease. The contribution of lymphocytic infiltration to exocrine gland dysfunction is, however, still poorly understood and needs further investigation. A challenge for the future is to treat patients as early as possible to prevent damage to the exocrine glands, which is apparently irreversible once initiated. Another challenge for future research is to unravel patient heterogeneity, possibly by the detection of clinical and molecular disease phenotypes, to enable personalized treatment of pSS patients.

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11

NEDERLANDSE SAMENVATTING

DANKWOORD

CURRICULUM VITAE

LIST OF PUBLICATIONS

NEDERLANDSE SAMENVATTING

Het syndroom van Sjögren is een chronische, systemische auto-immuunziekte, die gekenmerkt wordt door infiltratie van lymfocyten in de exocriene klieren, in het bijzonder de speekselklieren en traanklieren. De meest voorkomende symptomen van het syndroom van Sjögren zijn een droge mond, droge ogen en chronische vermoeidheid. Naast de exocriene klieren kunnen ook andere organen aangetast worden, wat de systemische aard van deze ziekte benadrukt. Hyperactivatie van B lymfocyten (B cellen) lijkt een belangrijke rol te spelen in het ziekteproces van het syndroom van Sjögren. De B cellen zijn daarom een interessant doelwit voor behandeling. Ook T lymfocyten (T cellen) lijken een rol te spelen in het ziekteproces, onder andere door hulp te geven aan de B cellen. Het onderzoek wat beschreven wordt in dit proefschrift heeft twee doelen: (1) Het identificeren van T en B cel-gerelateerde biomarkers die de aanwezigheid en ziekteactiviteit van het syndroom van Sjögren kunnen voorspellen en (2) het analyseren van de effecten van immunotherapie met rituximab en abatacept op T cel afhankelijke B cel hyperactiviteit in patiënten met het syndroom van Sjögren.

Deel 1: T cel afhankelijke B cel hyperactiviteit: biomarker voor ziekte?

In het eerste deel van dit proefschrift wordt de relevantie van meerdere T cel en B cel gerelateerde biomarkers bij dit ziektebeeld beschreven. We hebben gekeken of deze biomarkers de aanwezigheid van het syndroom van Sjögren kunnen voorspellen, en of ze gebruikt kunnen worden om ziekteactiviteit te monitoren. In **hoofdstuk 2** hebben we de huidige kennis samengevat over de rol van T helper 17 (Th17) cellen bij het syndroom van Sjögren. Th17 cellen behoren tot een bepaalde T cel subset die interleukine 17 (IL-17) produceert en deze cellen lijken betrokken bij verschillende auto-immuun- en chronische ontstekingsziekten. In deze context hebben we ook de plasticiteit van deze subset (het vermogen om van fenotype en functie te veranderen) bediscussieerd. We concluderen dat Th17 cellen betrokken zijn bij het lokale ontstekingsproces in patiënten met het syndroom van Sjögren via pro-inflammatoire effecten op de epitheelcellen en via het bevorderen van de vorming van lymfoïd weefsel in de speekselklieren zelf, het zogenaamde ectopisch lymfoïd weefsel. De bijdrage van Th17 cellen aan systemische ziekteactiviteit (buiten de klieren) is echter niet zo evident. De plasticiteit van deze subset zou hier mede aan ten grondslag kunnen liggen, omdat de cellen op verschillende manieren gedefinieerd worden en studies daardoor lastig te vergelijken zijn. Het is aannemelijk dat plasticiteit richting Th17.1 cellen, die zowel IL-17 als interferon-γ produceren, het chronische ontstekingsproces en B cel activatie in de speeksel- en traanklieren van patiënten met het syndroom van Sjögren zal bevorderen.

Naast Th17 cellen hebben we ook een T cel subset bestudeerd die gespecialiseerd is in het geven van hulp aan B cellen, de zogenaamde T folliculaire helper (Tfh) cellen.

In de **hoofdstukken 3a** en **3b** worden studies beschreven naar de prevalentie en het fenotype van Tfh cellen in bloed van patiënten met het syndroom van Sjögren. Ook de suppressieve tegenhanger van Tfh cellen, de zogenaamde T folliculaire regulatoire (Tfr) cellen, werden bestudeerd. We hebben laten zien dat de relatieve aantallen van zowel Tfh als Tfr cellen verhoogd zijn in het bloed van patiënten met Sjögren ten opzichte van sicca controles (patiënten met klachten van droge mond en/of ogen, die niet voldoen aan de classificatie criteria voor Sjögren) en gezonde individuen. De Tfr cellen waren zelfs sterker verhoogd dan Tfh cellen, wat resulteerde in een verhoogde Tfr/Tfh ratio in bloed van patiënten ten opzichte van sicca controles en gezonde individuen. De relatieve aantallen van Tfh en Tfr cellen in bloed waren geassocieerd met de serum concentratie van IgG en CXCL13, twee biomarkers die gerelateerd zijn aan T cel afhankelijke B cel hyperactiviteit. Daarnaast waren deze cellen gecorreleerd aan de mate van systemische ziekteactiviteit, die gemeten werd met de EULAR Sjögren's syndrome systemic disease activity index (ESSDAI) score. De resultaten van deze studies laten zien dat de relatieve aantallen van Tfh en Tfr cellen in het bloed bruikbare biomarkers zijn voor systemische ziekteactiviteit bij het syndroom van Sjögren. De positieve correlatie tussen Tfr cellen, B cel activiteit en ziekteactiviteit is echter opmerkelijk. Tfr cellen zouden een buitensporige afweerrespons, zoals bij het syndroom van Sjögren, namelijk moeten onderdrukken. Een mogelijke verklaring voor de verminderde suppressie door Tfr cellen komt voort uit onze bevinding dat regulatoire T cellen, Tfr cellen in het bijzonder, een lagere expressie hebben van de inhibitoire receptor CTLA-4.

In de volgende twee hoofdstukken hebben we specifiek gekeken naar B cel gerelateerde biomarkers. De studie beschreven in **hoofdstuk 4** had als doel het karakteriseren van een B cel subset die geassocieerd is met het epitheel en de receptor FcRL4 tot expressie brengt. De aanwezigheid van deze B cellen rondom en in het epitheel van de uitvoergangen in de speekselklieren kan gezien worden als een karakteristiek kenmerk bij patiënten met Sjögren. Bovendien zijn deze FcRL4-positieve B cellen mogelijke voorloper cellen van mucosa-geassocieerd lymfoïd weefsel (MALT) lymfoom, een type B cel lymfoom wat zich ontwikkelt in 5-10% van de patiënten, meestal in de oorspeekselklier. De aanwezigheid van grote aantallen FcRL4-positieve B cellen in de oorspeekselklier kan mogelijk patiënten identificeren die een groot risico hebben op MALT lymfoom ontwikkeling. Om deze cellen te karakteriseren in patiënten met Sjögren, voordat ze een lymfoom ontwikkelen, hebben we FcRL4-positieve B cellen geïsoleerd uit speekselklier weefsel. Vervolgens hebben we het gen expressie profiel van deze cellen in kaart gebracht. We observeerden verhoogde expressie van genen die betrokken zijn bij migratie naar de weefsels en cel adhesie, wat overeenkomt met de locatie van deze cellen dichtbij het epitheel. Verder zagen we een verhoogde expressie van genen die een rol spelen bij ontsteking en de overleving van B cellen. Aan de hand van deze eigenschappen concluderen we dan FcRL4-positieve B cellen een bijdrage

kunnen leveren aan de epitheelschade die gezien wordt in de speekselklieren van patiënten met Sjögren. Daarnaast laten onze resultaten zien dat deze cellen mogelijk vatbaar zijn voor mutagenese. In combinatie met een hoge delingsactiviteit, zouden deze cellen zich relatief frequent kunnen ontwikkelen tot tumor cellen.

In navolging van FcRL4-positieve cellen als mogelijke biomarkers voor MALT lymfoom, laten we in **hoofdstuk 5** zien dat in 50% van de patiënten met MALT lymfoom in de speekselklier een afwijkende ratio van immunoglobuline vrije lichte ketens (κ/λ) in serum aanwezig is, waarbij κ sterker verhoogd is dan λ. In patiënten met Sjögren zonder MALT lymfoom zijn zowel κ als λ vrije lichte ketens frequent verhoogd, maar de ratio is zelden afwijkend. We concluderen dat de ratio tussen κ en λ vrije lichte ketens in serum een bruikbare biomarker is voor de aanwezigheid van MALT lymfoom, en te gebruiken is in combinatie met conventionele biomarkers, zoals cryglobulinemie, lymfopenie, complement verbruik en aanhoudende zwelling van de speekselklier(en). In **hoofdstuk 5** laten we ook zien dat de waardes van vrije lichte ketens in serum, met name van het κ type, gebruikt kunnen worden om het effect van immunotherapie op B cel activiteit te monitoren.

Deel 2: T cel afhankelijke B cel hyperactiviteit: doelwit voor behandeling?

In het tweede gedeelte van dit proefschrift hebben we het effect van immunotherapie met rituximab of abatacept op T cel afhankelijke B cel hyperactiviteit in patiënten met Sjögren bestudeerd. In de studie beschreven in **hoofdstuk 6** laten we zien dat B cel depletie therapie met rituximab naast de te verwachten effecten op B cellen, ook effecten heeft op de circulerende T cellen. Van alle T cellen in bloed waren in het bijzonder de Tfh cellen aangedaan, waarbij de verhoogde relatieve aantallen van deze cellen tijdens de behandeling normaliseerden tot waardes die gezien worden in gezonde individuen. De afname van circulerende Tfh cellen was geassocieerd met de verbetering van meerdere objectieve maten van ziekteactiviteit, inclusief de eerder genoemde ESSDAI score. In **hoofdstuk 7** hebben we de huidige literatuur over de klinische en biologische effecten van rituximab behandeling in patiënten met Sjögren samengevat en bediscussieerd. We concluderen dat rituximab in een subgroep van patiënten gunstige effecten heeft op B cel activiteit, de morfologie van de speekselklieren, droogheid, vermoeidheid en meerdere extraglandulaire manifestaties. De literatuur laat verder zien dat patiënten met gematigde tot ernstige systemische ziekteactiviteit, d.w.z. activiteit in meerder domeinen van de ESSDAI score, het meeste baat zouden hebben bij rituximab behandeling. Naast immunotherapie gericht op B cellen, heeft de biological abatacept (gericht op het verhinderen van T cel activatie) ook gunstige klinische effecten laten zien in patiënten met Sjögren. In **hoofdstuk 8** beschrijven we de effecten van abatacept op T cellen en T cel afhankelijke B cel activatie in deze patiënten. Behandeling met abatacept

zorgt voor een verlaging van het aantal Tfh cellen in bloed, evenals een verlaagde expressie van de activatie marker ICOS op T cellen. De verlaagde expressie van ICOS zagen we zowel in het bloed als in de speekselklieren. De daling in ICOS expressie op de overgebleven circulerende Tfh cellen correleerde longitudinaal met de daling in ESSDAI scores. B cel activiteit werd ook verminderd door abatacept, wat zich uitte in een daling van plasmablasten en autoantistoffen in bloed.

Als laatste laten de resultaten beschreven in **hoofdstuk 9** zien dat behandeling met abatacept ook zorgt voor een verlaagde expressie van Bruton's tyrosine kinase (BTK) in B cellen van behandelde patiënten. BTK speelt een belangrijke rol in de signalering via de B cel receptor en daarmee in B cel proliferatie en overleving. Vóór behandeling was de expressie van BTK verhoogd in het merendeel van de Sjögren patiënten en dit correleerde met waardes van autoantistoffen (reumafactor) in serum en met de hoeveelheid T cellen in de oorspeekselklier. Samen met de bevindingen beschreven in hoofdstuk 8 laten deze resultaten zien dat de interactie tussen B cellen en T(fh) cellen een centrale rol speelt in het ziekteproces van het syndroom van Sjögren.

Conclusies

Het bestuderen van de effecten van immunotherapie bij het syndroom van Sjögren heeft in sterke mate bijgedragen aan het begrijpen van de pathogenese van deze aandoening. Het onderzoek beschreven in dit proefschrift heeft aangetoond dat de frequentie van circulerende Tfh cellen een adequate biomarker voor ziekteactiviteit is. Lokaal in de speekselklieren lijken echter ook andere effector T cel subtypes en B cellen een belangrijke bijdrage te leveren aan het ontstekingsproces. In dit proefschrift beschrijven we ook dat de ratio van immunoglobuline vrije lichte ketens (κ/λ) in serum een bruikbare biomarker is voor de aanwezigheid van MALT-lymfoom. Verder tonen we verschillende biologische effecten aan van immunotherapie met rituximab of abatacept bij het syndroom van Sjögren. Met name circulerende Tfh cellen en hyperactiviteit van de B cellen worden verminderd door deze immunotherapie. Aan de hand van onze resultaten kunnen we veronderstellen dat het remmen van de interactie tussen T cellen en B cellen cruciaal is voor succesvolle behandeling van (systemische) ziekteactiviteit bij het syndroom van Sjögren.

DANKWOORD

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CURRICULUM VITAE

Gwenny Matthea Petronella Johanna Verstappen (1988) was born and grew up in Nijmegen, the Netherlands, where she finished high school (Stedelijk Gymnasium Nijmegen) in 2006. That same year she started her pharmacy studies at the University of Groningen and obtained her Master's degree in Pharmacy in 2013. During her studies she was an active member of the Pharmaceutical Student's Association "Pharmaciae Sacrum" where she was President in 2010-2011. She specialized in immunology during her Master thesis at the department of Rheumatology & Clinical Immunology (University Medical Center Groningen). After graduating, she continued her research and immunology training as a PhD student under the supervision of prof. dr. H. Bootsma, prof. dr. F.G.M. Kroese, and prof. dr. A. Vissink. Her PhD thesis focused on T cell-dependent B cell hyperactivity in patients with primary Sjögren's syndrome. She presented her work at several national and international conferences like the European League Against Rheumatism (EULAR), the American College of Rheumatology (ACR), the International Symposium on Sjögren's Syndrome (ISSS), the Germinal Centre Conference (GCC), and the Dutch Society for Rheumatology (NVR) annual meeting. In 2018, she was awarded a Rubicon grant from NWO to continue her research at the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia.

LIST OF PUBLICATIONS

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